

**DEVELOPING NOVEL APPROACHES TO TREAT TRAUMA-
ASSOCIATED FUNGAL INFECTIONS**

by

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ABSTRACT

Traumatic injuries often result in infections leading to high morbidity and mortality. Fungal spores, the point of interest of this research, of species such as *Mucormycetes*, *Scedosporium*, *Lomentospora*, *Fusarium* and *Candida*, are ubiquitous in the environment.

Here, we have taken dual approach to investigate: 1) how two novel treatments - acetic acid and blue light - are effective against those fungal species, and 2) how highly pathogenic and antifungal resistant species of *Scedosporium* and *Lomentospora* interact with innate immune cells.

Firstly, I have demonstrated that low concentrations of acetic acid can inhibit spore germination as well as prevent growth of all fungal species tested. Therefore, acetic acid can be used as a potential preventative measure and/or treatment on infected wounds. Secondly, I have shown that blue light irradiation is effective against some, but not all fungal species, and should therefore be used with caution in a healthcare setting.

In the latter part of this thesis I characterize the *Scedosporium* and *Lomentospora* interaction with macrophages. Using this system, I show for the first time that phagocytosis and lateral transfer are two distinct events and that these neglected fungal pathogens may hold interesting promise as a tool for understanding the fungal/host interaction. Lastly, I report on our preliminary attempts to establish a novel *ex-vivo* skin model and show that Langerhans cells within the epidermis do not mature nor release TNF α cytokine following infection with *R. microsporus* and *S. apiospermum*.

To

Gabrielle and Grzegorz

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ABBREVIATIONS

Amp B	amphotericin B
ATP	adenosine-5'-triphosphate
BCECF-AM-	[2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester
BL	blue light
BMM	bone marrow macrophages
BSA	bovine serum albumin
CF	cystic fibrosis
CFU	colony forming unit
CLR	c-type lectin receptor
CNS	central nervous system
cRPMI	complete RPMI media 1640
CSH	cell surface hydrophobicity
DC-SIGN	dendritic cell-specific intracellular adhesion molecule-3-Grabbing Non-integrin
dDC	dermal dendritic cells
DENV	Dengue virus
DMEM	dulbecco's modified eagle medium
DMSO	dimethyl sulfoxide
DoD	Department of Defense Trauma Registry
EDTA	ethylenediaminetetraacetic acid
ELWD	extra long working distance
FBS	fetal bovine serum

FSC	forward scatter
G-CSF	granulocyte colony-stimulating factor
GlcCer	glucosylceramides
GM-CSF	granulocyte macrophage colony-stimulating factor
GPI	glycosyl phosphatidyl inositol
GPR78	the glucose-regulated protein 78
HBRC	human biomaterials resource centre
HMDMs	human monocyte-derived macrophages
HMG	high mobility group
H1 VLP	influenza virus-like particle vaccine
ID	intradermal injection
IDEC	inflammatory dendritic epidermal cells
IFI	invasive fungal infection
inf DC	inflammatory DCs
IP-10	interferon gamma-induced protein
ITAM	immunoreceptor tyrosine-based activation motif
ITC	itraconazole
LC	langerhans cells
LDM	inhibition of lanosterol 14 α - demethylase
LLO	pore-forming toxin expressed by <i>L. monocytogenes</i>
mAb	monoclonal antibody
Mac	macrophage
MBL	mannose binding lectin
MCP	monocyte chemoattractant protein

M-CSF	macrophage colony-stimulating factor
MEC	minimum effective concentration
MIC	minimum inhibitory concentration
MIP	macrophage inflammatory protein
MNCs	mononuclear leukocytes
mo-Mac	monocyte-derived macrophage
mono	monocytes
MR	mannose receptor
MyD88	myeloid differentiation primary response 88
NET	neutrophil extracellular trap
NLR	NOD-like receptor
OD	optical density
PAMP	pathogen associated molecular pattern
PBS	phosphate- buffered saline
pDC	plasmacytoid DCs
PDT	photodynamic therapy
PFA	paraformaldehyde
PMNs	human polymorphonuclear leukocytes
PRBC	Packed red blood cells
PRM	peptidorhamnomannan
PRR	pattern recognition receptor
PSC	posaconazole
RBC	red blood cell lysis
RES	reduced echinocandin susceptibility

ROS	reactive oxygen species
rpm	revolutions per minute
RPMI	Roswell Park Memorial Institute
SSC	side scatter
TCR	calcineurin inhibitor tacrolimus
TDM	therapeutic drug monitoring
TIDOS	The Trauma Infectious Disease Outcomes Study
TipDC	TNF alpha and iNOS producing DC
TLR	toll-like lectin receptor
TNFα	tumor necrosis factor alpha
TPT	triose phosphate transporter
VA	Department of Veterans Affairs
VRC	voriconazole
YPD	yeast peptone dextrose
17AAG	Hsp90 inhibitor 17-demethoxy-17-(2-propenylamino) geldanamycin
β-TRIF	TIR domain-containing adapter-inducer interferon β

1. INTRODUCTION

1.1 Trauma-associated wounds

Human skin is a protective surface, whose role is to protect the host by regulating microbial populations living on the skin and resisting invasion by potential environmental pathogens (Bowler et al., 2001) whilst preserving body fluid homeostasis and thermoregulation (Church et al., 2006). When this barrier is breached, and tissue loses its integrity, the resultant wound forms an ideal environment for microbial colonization and invasion. The wound can be caused by traumatic injuries sustained in both military and civilian environments like agricultural, motor vehicle, blunt crush injuries and natural disasters (Warkentien et al., 2012). Wounds can be divided broadly into acute or chronic classes. Acute wounds such as surgical wounds, burns, cuts or traumatic injuries, are a major health problem, with 11 million people affected, and approximately 300,000 people hospitalised yearly in the United States alone (Demidova-Rice et al., 2012). Chronic, non-healing wounds such as diabetic ulcers cost the National Health Service in the United Kingdom in 2005 - 2006 £15 -18 million annually (Nunan et al., 2014). The wound nutritive, moist and warm environment provides an ideal ecosystem for various pathogens such as bacteria, fungi and viruses. Our immune system activates cells within minutes of the injury when a pathogen is recognised (Hazeldine et al., 2017). Neutrophils and macrophages are the first line of defense after traumatic injury. However, chronic, non-healing wounds are very often hypoxic, with poor blood perfusion resulting in cell death and tissue necrosis, further supporting microbial growth (Bowler et al., 2001). In addition, chronic wounds can restrict the delivery of antibiotics due to critical blood supply and adequate

perfusion of target tissue, making treatment extremely difficult (Nunan et al., 2014). Wound colonization is most commonly polymicrobial. Common aerobic pathogens such as *Staphylococcus aureus* and *Pseudomonas aeruginosa* are the primary cause of delayed healing in acute and chronic wounds (Bowler et al., 2001). However, anaerobes, which often die in the presence of air, such as *Bacteroides*, *Prevotella*, *Porphyromonas*, or *Peptostreptococcus* spp. frequently survive in wounds. However, it is not only bacteria that are able to survive within wounds, fungal pathogens can also. With many traumatic injuries occurring outdoors, fungal contamination from environmental sources is a serious consideration for treatment of these injuries. It is these fungi that infected traumatic wounds that will be the focus of this thesis.

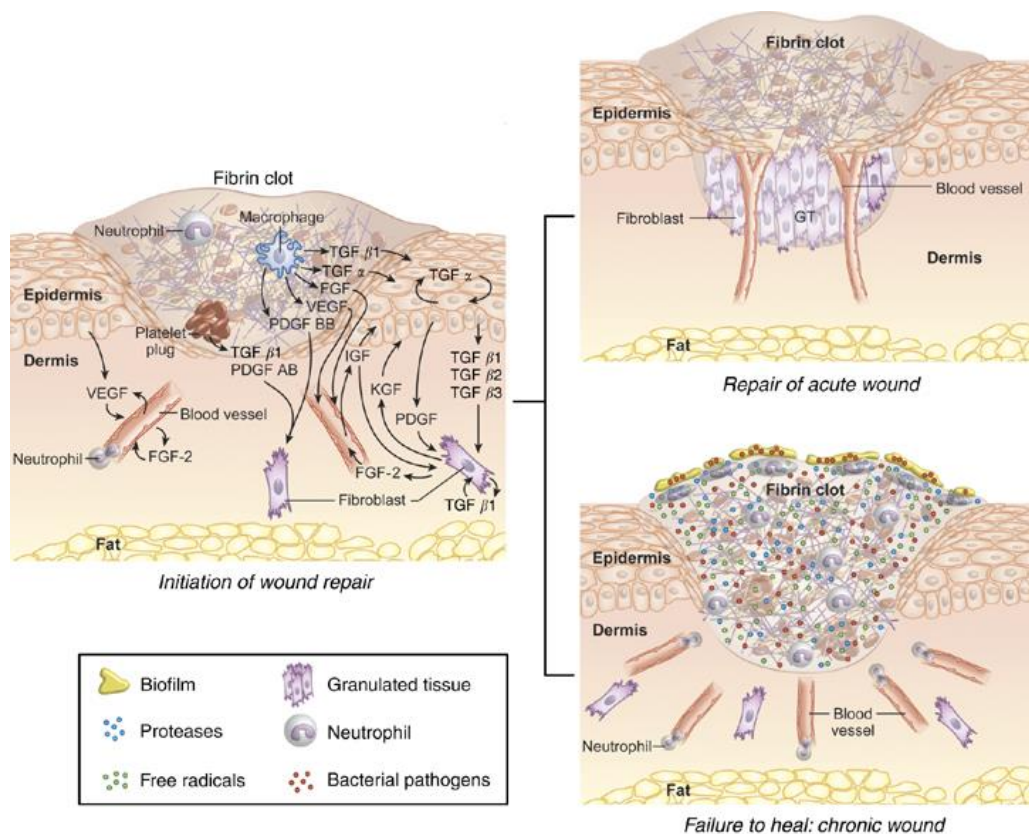


Figure 1.1 Acute versus chronic wound.

Figure adapted from Clark et al., 2007. Left panel presents tissue injury followed by blood clotting, platelet aggregation and migration of phagocytic cells that attempt to kill microorganisms. Macrophages secrete growth factors into the wound environment. Then fibroblast and endothelial cells express integrins to migrate and secrete growth factors, as do epidermal cells. Under normal physiological conditions (upper right panel), the wound continues to heal. Migrating epidermis now travels over newly formed tissue. In repair settings, proteases are crucial for invasion of the clot and ingrowth of the wound. Platelets, macrophages, epidermal and endothelial cells and fibroblasts secrete many growth factors. When microbial invasion interrupts wound healing (lower right panel), venous failure results in fluid transudation and fibrin cuffing. Microorganisms very often produce biofilms that protects colonies of mixed organisms, resisting both phagocytic cells and antibiotics (Clark et al., 2007).

1.2 Clinical epidemiology of trauma-associated fungal wound infections

In most cases of trauma-associated fungal infections, fungi are introduced into the wound as spores from environmental contamination, which are then able to germinate and spread. This is particularly problematic in a range of settings that I will now discuss further.

War

It is well known that military wounds are typically different from civilian wounds in terms of the mechanism of injury and level of contamination (Evriviades et al., 2011). During recent conflicts in Afghanistan or Iraq, many patients had wounds heavily contaminated with environmental fungi such as *Mucormyces* spp., *Fusarium* spp., *Aspergillus* spp., *Penicillium* spp., or *Chaetomium* spp. (Evriviades et al., 2011).

Rodriguez et al. (2014 a, b) have collected data for the Trauma Infectious Disease Outcome Study (TIDOS) about US military personnel from the Department of Defense (DoD) Trauma Registry and the Department of Veterans Affairs (VA) (Rodriguez et al., 2014 a, b). They have described that Invasive Fungal Infections (IFIs) associated with traumatic injuries tend to be associated with a morbidity and mortality rate as high as 11-38 %. IFI has also been associated with a higher rate of lower extremity amputations (79 % in comparison to 46 % in a control group of patients), and those infections were more hypotensive (low blood pressure) and acidotic (increased acidity in the blood and other tissues) than a control group. Patients that have developed IFI have also received larger volumes of packed red blood cell (PRBC) transfusion (median 29 U), compared to the control group (11 U) (Rodriguez et al., 2014 a). Overall, the patient group with recurrent wound necrosis were infected mainly with *Mucormyces* spp. (11 %) and all have been treated with antifungal ((liposomal amphotericin B (ampB)) for about 5 days (Rodriguez et al., 2014 b). Tribble & Rodriguez (2014) have reviewed US military patients that have been infected with fungal pathogens during the Iraq and Afghanistan conflicts ranging over 2001-2011. Their research reveals that limb amputations following injuries resulted in 3.6 per 100 combat, with significant increase in number of amputations from 3.5 per 100 combat in 2010 to 14 in 2011 (Tribble & Rodriguez, 2014). Combats who had a traumatic injury usually undergo three washouts and/or debridement in the first 72 hours after injury, followed by wound assessment and continued debridement within first 12 hours on arrival to USA. Similarly to Rodriguez et al. (2014 a, b), Tribble & Rodriguez (2014) links risk factors of IFI to substantial blood transfusion which might have an immunosuppressive effect and supply of iron, associated with *Mucormyces* spp. infections (Tribble & Rodriguez,

2014). Among fungal species that caused infections in US military patients suffering from traumatic wound injuries, *Mucormycetes* are the predominant species isolated from 35 % of cases (*Mucor* spp. 62%, *Rhizopus* spp. 4%, *Apophysomyces elegans* 5%, *Saksenaia vasiformis* 14%), then *Aspergillus* spp., *Fusarium* spp., *Scedosporium* spp. Blyth et al. (2014) have also presented data showing *Candida* spp. to be associated with wounded military personnel with mortality rate of 7.1 %, however, they state no medical evidence that this mortality is due to *Candida* infection (Blyth et al., 2014). Mitchell et al., (2014) have reported on mucormycosis infections (infections caused by species belonging to order *Mucormycetes*) from January 2003 to November 2009, presented in an adult burn center in Huston (United States), and found 2.4 per 1000 admissions were due to traumatic or burn injury (including patients injured in Iraq or Afghanistan during war). While, Warkentien et al. (2012) reported that 83.3 % of combat patients had at least 1 fungal species isolated from their wound(s) from June 2009 to December 2010. More specifically, those fungi isolated were identified as *Mucormycetes* spp., *Fusarium* spp. and/or *Aspergillus* spp. Kronen et al. (2017) research shows post-traumatic IFI in military personnel caused by *Mucormycetes*, *Aspergillus* and *Fusarium* spp., 34%, 31% and 22% respectively. A wide diversity of *Aspergillus* spp. infect such wounds, including: *A. terreus* 10%, *A. flavus* 9% and *A. fumigatus* 1%. Other fungal species associated with military wound infections were *Lichtheimia*, *Apophysomyces*, *Aspergillus niger*, *Bipolaris*, *Penicillium*, *Alternaria*, *Scedosporium*, *Graphium*, *Candida* spp. (Kronen et al., 2017). This research also confirms others risk factors associated with IFI wound injuries, such as extensive blood transfusion, blast injury, dismounted status and above-knee amputations. McDonald et al. (2018) research highlights that many military personnel from the wars in Iraq and

Afghanistan remain at significant risk for trauma-related infections for many years following injury. 38% of enrolled patients developed new trauma-related infections after their initial hospital discharge and 29% of such infections occurred after leaving military service (McDonald et al., 2018). All the data summarised above by different research groups are similar in terms of morbidity and mortality rate associated with traumatic injuries, risk factors and fungal species that are linked with IFI.

Weintrob et al. (2015) have investigated epidemiology and clinical classification of traumatic wounds of 1133 US combat personnel injured during service in Afghanistan (2010-2011), among which 6.8 % had IFI. Their research usefully describes the classification of IFI, which is often lacking in other reviews. For a proven IFI case they describe patients with confirmed angioinvasion of fungal elements on histopathology samples, and describe probable IFI as those with fungal elements identified from histopathology without angioinvasion (Weintrob et al., 2015). "Possible IFI" were all cases in which mould has grown in tissue, however fungal elements were negative in histopathology, and those that had wound tissue with grown mould but histopathology was not sent for evaluation were classified as an unclassifiable IFI (Weintrob et al., 2015). Hospitalization in the case of proven/probable cases was around 36-71 days, while for the possible/unclassifiable cases that time duration lasted for 29-57 days. 22 % of patients with proven/probable cases had amputations in comparison to possible/unclassifiable cases, whose amputation rate was 13 % (Weintrob et al., 2015). 16 % of IFI patients did not receive any antifungals, and those who have received dual antifungal treatment (usually liposomal amphotericin B and voriconazole) were proven/probable cases- 59 % versus 44 % in possible/unclassifiable cases.

Mortality rate was 9.3 % vs 4.3 % in proven/probable cases vs possible/unclassifiable cases respectively (Weintrob et al., 2015).

The type of infection and mortality rate of military patients are greatly associated with environmental factors, such as temperature and humidity. Tribble et al., (2015) have described environmental characteristics, risk factors of injury and risk of developing IFI after combat trauma in Afghanistan in 2009-2011. For instance, military personnel in southern Afghanistan exhibited an increased risk of developing IFIs relative to those from the eastern region (61% vs. 20% respectively). This likely reflects the fact that the southern region was much warmer, with higher isothermality (constant temperature) and humidity than the eastern region, thus supporting higher levels of fungal growth. Similarly, in Iraq, where environmental conditions are comparable to southern Afghanistan, there is also a higher frequency of fungal wound contamination (Tribble et al., 2015).

Traumatic wound infections caused by combat injuries are very common. Delayed diagnosis at field hospitals and time needed for patients transport very often ends with extensive surgical debridement and/or a high mortality rate. Therefore, there is a need for an easy to apply, effective and cheap treatment that can be used at the site and time of the injury.

Environmental and man-made disasters

During environmental disasters such as tsunami or earthquakes, survivors often receive wounds with high levels of environmental contamination. Amid wounded survivors of tsunami disasters, around two-third of patients' wounds become infected in the first 72 hours (Wuthisuthimethawee et al., 2015). Those wounds tend to be contaminated with environmental pathogens and foreign matter, the crushed and dead

tissue providing a medium for bacterial and fungal growth. Following the 2004 tsunami disaster in Southeast Asia, patient's wounds were contaminated with both sea and fresh water, soil, sewage, and various foreign matter containing environmental pathogens that were very problematic to treat (Maegerle et al., 2006). Factors such as delayed wound cleaning, patient transport to hospital and primary wound closure are very often a reason for subsequent deep surgical debridement and an associated high mortality rate. Traumatic injuries are also very common in vehicle accidents or agricultural injuries (Vitrat-Hincky et al., 2009). Near-drowning accidents are also case of invasive fungal infections, especially of *S. apiospermum* (He, X.H. et al. 2015). Similarly to military patients, civilian traumatic injuries are most commonly infected with fungal pathogens such as *Mucormycetes*, *Scedosporium*, *Aspergillus* and *Fusarium* species (Kronen et al., 2017). Post-traumatic filamentous fungal infections are particularly associated with traffic (41 %) and agricultural (25 %) accidents as well as natural disasters (12 %) (Kronen et al., 2017). Overall, civilians often receive a delayed diagnosis compared to military personnel, perhaps due to postponement in sampling. Diagnosis following a tornado in Joplin (Missouri) ranged from 10 to 19 days (time from sample collection to diagnosis) (Kronen et al., 2017). Risk factors for IFI in civilian patients affected with traumatic injuries are wound contamination by soil, plant matter and gravel. Wearing synthetic fabric at the time of injury might also support IFI due to the moisture and warmth of the skin in comparison with cotton clothes (Kronen et al., 2017). Overall mortality rate for trauma-associated fungal infected civilian patients is 25-41 %, and most occur within first 2 weeks of hospitalization (Kronen et al., 2017).

1.3 Introduction to trauma associated fungal pathogens

Mucormycetes

Life cycle. *Mucormycetes* spp. belongs to the phylum Glomeromycota and subphylum Mucormycotina (Müller et al., 2018). Within the phyla, the order *Mucormycetes* contains the genera *Mucor*, *Rhizomucor*, *Rhizopus* and *Lichtheimia* (Kauffman, 2004), which, together, cause most cases of human mucormycosis. These fungal species reproduce both sexually and asexually. In the asexual reproduction, on the apex of sporangiophore, a sporangium is present (Mendoza et al., 2014). Sporangia contain sporangiophores which disperse and then germinate to produce a mycelial complex in favourable environmental conditions. During their sexual development, two mating types of hyphae (+) and (-), sense each other, followed by gamete fusion that forms zygosporangia (Mendoza et al., 2014). These zygosporangia have the ability to germinate and form a sporangium (at the apex) containing sexual meiospores (Mendoza et al., 2014). Xu et al. (2017) have shown that sexual reproduction coupled with meiosis and recombination plays a role in virulence and pathogenicity. It is well known that during fungal sexual reproduction, different mating types are required to form gametes/sexual fusion (Xu et al., 2017). This process is controlled by a genomic region known as the mating type locus (*MAT*). In *Mucormycetes* spp., mating is regulated by divergent alleles of a single gene (*SexM/SexP*). This gene consists of high mobility group (HMG) transcription factor gene between genes encoding phosphate transporter homologue (TPT) and RNA helicase (Xu et al., 2017).

Infection. The past two decades have shown an increase in patients with mucormycosis due to many underlying diseases (Pfaller and Diekema, 2004). Such an

increase in mucormycosis is associated with a high morbidity and mortality rate and is the result of an increased patient population at risk from the development of serious fungal infections (Pfaller and Diekema, 2004). The mortality rate associated with patients suffering from mucormycosis can be as high as 40-70 % (Müller et al., 2018). For example, in France, mucormycosis represents 1.5 % of 35876 cases with invasive fungal infections with an increase of 7.3 % per year (2006) (Lortholary, Fernández-Ruiz & Perfect, 2016). Haematological malignancy patients as well as the healthcare associated outbreaks represented 50-70 % of mucormycosis cases in 2016, while diabetic patients 20 % of mucormycosis in Europe and USA (Lortholary, Fernández-Ruiz & Perfect, 2016). At risk patients are those who have received organ or stem cell transplant, patients with diabetes mellitus, malignancy or neutropenia and those who receive deferoxamine therapy (therapy used to remove excess of iron from the body e.g. to treat hemochromatosis) or injection drug users (Roden et al., 2005). *Rhizopus arrhizus* is the most common species isolated from clinical samples (Müller et al., 2018). Though, there are also cases of mucormycosis in patients that are not immunocompromised (Venkatesh et al., 2018; Dogra, Bhutani and Gupta, 2018). As *Mucormycetes* are pervasive environmental fungi found in soil and decayed wood, injuries that introduce such environmental material into the wound are at a higher risk of developing necrotizing cutaneous mucormycosis. *Mucormycetes* spp., are the third emerging group of fungal pathogens related to post-trauma infection, representing 20 % of cases (Lortholary, Fernández-Ruiz & Perfect, 2016). Epidemiological studies show that varying underlying conditions are subjected to different localisations of mucormycosis, for example patients with organ transplant are in risk of getting infection of that organ; victims of traumatic injury of the infection of the side of wound; or

agricultural workers, nasal or brain infection followed by spore inhalation (Tacke et al., 2014).

Virulence traits. Morace & Borghi (2012) have reviewed virulence and pathogenesis caused by *Mucormycetes* spp. and describe their iron association, and their ability to acquire iron from host with enhanced growth and hyphal development of *Mucormycetes* spp. *in vitro* and their increased pathogenicity *in vivo*. A novel host receptor GPR78 (the glucose-regulated protein 78), was shown to be required for pathogenesis of *Rhizopus oryzae* in diabetic mice (Liu, M. et al., 2010). They presented that high glucose and iron presence, regulate increase expression of GPR78 causing endothelial cells damage, however, the fungal ligand is unknown (Liu, M. et al., 2010).

Xu et al. (2017) research on 17 clinical isolates of *Mucor irregularis* from patients in China, showed that (+) mating type isolates caused greater lesion and more damage than the (-) mating type isolates. They have concluded from that research that mating types might have a correlation with the severity of inflammation, although it may not be directly linked to a virulence factor, the *M. irregularis* had a different level of immune response (especially Th-1 and Th17 pathway) to different mating type (Xu et al., 2017). They have also compared their results to others, stating that (+) mating type had an increased pathogenicity in *Mucor piriformis* when used as inoculum in plant causing larger lesion than (-) mating type of this isolate (Michailides & Spotts., 1986); or (+) mating type of *Mucor amphibiorum* is able to cause severe infection in toads in comparison to (-) type (produced spherules more rapidly leading to better dissemination) (Stewart & Munday., 2005). In contrast (-) mating types of *Mucor circinelloides* *F. lusitanicus* (Mcl) were more virulent in the wax moth host in

comparison to (+) mating types, however, the loss of virulence in (-) mating types was correlated to the sporangiospore size not necessarily to the *MAT* locus (Li et al., 2011).

Scedosporium, Lomentospora

Life cycle. *Scedosporium* and *Lomentospora* spp. are pervasive environmental fungi, generally found in soil, polluted water, sewage, as well as indoor air (Cortez et al., 2008). Similarly to *Mucormycetes* spp., they reproduce sexually and asexually. Telomorph (sexual reproductive stage) of *Scedosporium/Lomentospora* spp. strains spontaneously produce fruit bodies called ascigerous (Guarro et al., 2006). *P. boydii* (also called *S. apiospermum*), for example, produce cleistothecia (closed fruit bodies) which are yellow-brown to black coloured with spherical shape and size of 140-200 µm in diameter. When this are crushed, they release thin-walled, spherical, evanescent asci, which quickly dissolve to release 8 lemon-shape, golden-brown in colour ascospores, 4-5 x 5-7 µm in size (Guarro et al., 2006). The anamorph (asexual reproductive stage) is almost invariably present in *Scedosporium/Lomentospora* spp. It is composed of hyaline cylindrical conidiogenous cells growing of from undifferentiated hyphae. Those cells produce obovoidal, brown and sticky conidia, with size of 4-9 x 6-10 µm, from a short extensions (Guarro et al., 2006).

Infection. Within the genus *Scedosporium* there are two medically important species, *Scedosporium prolificans* (now renamed to *Lomentospora prolificans*) (Lackner et al., 2014) and *Scedosporium apiospermum*. *S. apiospermum* and *S. boydii* (previously *P. boydii*) have a worldwide distribution, while *L. prolificans* infrequently encountered in an environmental samples and used to appear more commonly in the climates of Australia and Spain, but more recently has been recognized in other European countries, USA and Korea (Ramirez-Garcia al., 2018). *Scedosporium* spp.

account for 25% of non- *Aspergillus* fungal infections (Campa-Thompson et al., 2014). The first reported case of human disease caused by *Scedosporium* was described in 1889 (otitis), and the infectious species was *P. boydii* (Cortez et al., 2008). Human infections caused by *Scedosporium*, *Lomentospora* spp. were initially primarily subcutaneous infections caused by trauma, which typically affected the feet of barefooted, native workers in India and so at that time the disease was called “Madura foot” after the region of Madura (India) where it was first identified (Cortez et al., 2008). However, in the early 1980s similar fungal infections started to occur in immunocompromised patients and today cases that disseminate to surrounding tissues or organs are not uncommon (Campa-Thompson et al., 2014; Torres-Sánchez et al., 2018). The fungus can cause airway abnormalities, including allergic bronchopulmonary reaction, by colonizing the respiratory tract, or disseminated diseases within skin and soft tissues such as ligaments or tendons. Other diseases caused by *Scedosporium*, *Lomentospora* spp. include: keratitis, otomycosis, septic arthritis, pneumonia, meningitis etc. (Cortez et al., 2008). Interestingly, *Scedosporium*, *Lomentospora* spp. infection is commonly found in near-drowning patients (i.e. those who have had extensive immersion underwater) especially if brain abscess or pneumonia has been diagnosed. There are no documented cases of *Scedosporium*, *Lomentospora* being transmitted from person to person (Campa-Thompson et al., 2014). Infections caused by *Scedosporium*, *Lomentospora* account for around 3% of all infections caused by fungi in post-transplant patients, with a mortality rate of 70% (Acharya et al., 2006). Ramirez-Garcia al. (2018) recently have recently summarized risk factors for *Scedosporium/Lomentospora* spp. infection such as: solid organ transplant, where *Scedosporium/Lomentospora* spp., infections are two-thirds of all

infections caused by those species; hematopoietic stem cell transplant; cancer and other immunodeficiencies (Ramirez-Garcia al., 2018). Central Nervous System (CNS) involvement in scedosporiosis (infection caused by species belonging to *Scedosporium* and *Lomentospora*) has been described including cases of solitary or multiple brain abscesses, meningitis and ventriculitis (Acharya et al., 2006). Scedosporiosis has also been stated to cause osteomyelitis (infection in a bone), endocarditis (inflammation of heart's inner lining), arthritis (joints disorder) and cystic bronchiectasis (bronchi or airways infection) in immunocompetent patients. Airway colonization by *Scedosporium* spp. in patients with cystic fibrosis starts during adolescence and become chronic in up to 54 % cases (Ramirez-Garcia al., 2018). German cystic fibrosis centre study presented by Schwarz et al. (2017), showed *Scedosporium-Lomentospora* infections to be 4 %, in France 8.6 %, Australia 17.4-25 %. They also presented that patients with those infection significantly reduced co-colonisation with *H. influenza* and with *Candida* spp., and increased co-colonisation with the mucoid phenotype of *P. aeruginosa* (Schwarz et al., 2017). There have also been cases of lung cancer patients with scedosporiosis and co-infection of *Aspergillus fumigatus* in cystic fibrosis patients (Hirschi et al., 2012). Bronchial infection by those species can lead to chronic inflammation or life-threatening invasive disease in case of lung transplant or hematological malignancy (Ramirez-Garcia al., 2018). *Scedosporium* but not *Lomentospora* spp. has also been linked to allergic bronchopulmonary mycoses that are about 3 % of all cases (Ramirez-Garcia al., 2018).

Virulence traits. Mello et al. (2018 a) have investigated the surface properties, adhesion and biofilm formation by different species of *Scedosporium* and *Lomentospora*. They showed that cell surface hydrophobicity (CSH) in *S.*

apiospermum, *S. minutisporum*, *S. aurantiacum* and *L. prolificans* influences adhesion and biofilm formation by those species. Zeta potential is the parameter that determines the electronic mobility of charged particle in a liquid media and represents the electronic double layer around the cell (Mello et al., 2018 a). This can be used to measure cell adhesion to the surfaces. Mello et al. (2018 a) have also measured electric charges of above mentioned species in context of melanin, and found that *L. prolificans* and *S. minutisporum* with higher electronegative charge were producing more melanin than *S. apiospermum* and *S. aurantiacum* that are less electronegative ones. They have also compared *Scedosporium/Lomentospora* ability to adhere to poly-L-lysine coated glass (positively charged) and showed enhanced adhesion in comparison to non-coated glass (Mello et al., 2018a). These strains have also produced distinct amount of adhesive mannose-rich molecules. Biomass and metabolic activity of *Scedosporium/Lomentospora* strains have reached the maximum after 72 hours, while production of extracellular matrix has been reached at 24 hours (Mello et al., 2018 a). Lastly, Mello et al. (2018 a) have checked whether *Scedosporium* and *Lomentospora* spp. can form biofilm on different medical devices, such as a nasogastric catheter made from a polyvinyl chloride, a late bladder catheter of siliconized latex, and a polyurethane nasoenteric catheter. All fungal species formed biofilms on all devices with *S. aurantiacum* and *S. minutisporum* formed thicker biofilms than *S. apiospermum* and *L. prolificans* (Mello et al., 2018 a).

Ramirez-Garcia al. (2018) review few virulence factors that has been published so far, such as: ability of conidiation (ability to produce spores) in host tissue by *L. prolificans*, that helps dissemination (Ortoneda et al.,2002); peptidorhamnomannan (PRM) that is expressed in both, conidial and hyphal cell wall, has been shown to be

related with adhesion and endocytosis by epithelial cells and macrophages, therefore, might facilitate virulence and dissemination (Pinto, M.R. et al., 2004; Barreto-Bergter, E. et al., 2008; Xisto, M.I. et al., 2016); α -glucan, found in *P. boydii* surface, has been shown to be essential conidial phagocytosis and induce cytokine secretion by immune cells involving TLR2, CD14 and MyD88 (Bittencourt, V.C.B. et al., 2006). More virulence traits summarized in the Ramirez-Garcia al. (2018) review are: glucosylceramides (GlcCer) (neutral glycosphingolipids) expressed by *S. apiospermum* are associated with growth, differentiation and infectivity of fungal cells (da Silva, A.F. et al., 2004; Pinto, M.R. et al., 2002); enzymes, such as Cu/Zn cytosolic superoxidase dismutase and monofunctional catalase from *P. boydii* are important for evasion of immune system (Lima, O.C. et al., 2007); melanin has also been shown to be involved in virulence by protecting fungus from UV, oxidative killing and H₂O₂, but not ampB (Al-Laaiby, A. et al., 2016).

Mello, T.P. et al. (2018 b) review also points out at few virulence traits of *Scedosporium/Lomentospora* spp., such as the fact that *Scedosporium/Lomentospora* spp. germination is influenced by at 37° C and CO₂ (5 %); or that the MIC value to different antifungals is always higher for conidia then a germinated conidia, supporting their survival and growth within host tissue (Mello, T.P. et al., 2018 b).

Fusarium

Life cycle. Similarly to the above mentioned fungal pathogens, *Fusarium* spp. also reproduce both, sexually and asexually, with haploid mycelial structures that are formed in both developmental stages (Dweba, C.C et al., 2017). Mycelial structures produce three types of mitotic spores during their asexual life cycle. These are: conidiophores that produce microconidia; sporodochium that produce macroconidia;

and hyphae within which chlamydospores are being produced (Dweba, C.C et al., 2017). During sexual life cycle, fungus grows as a haploid colony of hyphae and diploid stages preceding meiosis and the production of haploid, sexually produced spores called ascospores (Ma, L.J. et al., 2013). The flask shaped structure, called perithecium, contains a sac (ascus) that produce ascospores. Homothallic species are able to self-fertilize resulting in production of clonal ascospore progeny (apomixis), while heterothallic species are self-sterile (Ma, L.J. et al., 2013). Both sexual orientations result in airborne spores (Dweba, C.C et al., 2017).

Infection. Genus *Fusarium* consist of 70 species, with 12 causing human infections (Lortholary, Fernández-Ruiz, & Perfect., 2016). *Fusarium* spp. causes a distinctive range of infections, which include locally invasive, disseminated infections or superficial (onychomycosis, keratitis) infections (Nucci and Anaissie, 2007). Most superficial infections such as onychomycosis or fungal keratitis in immunocompetent individuals, or disseminated infections in immunocompromised patients with mortality rate up to 70 % in those cases (Lortholary, Fernández-Ruiz, & Perfect., 2016). *Fusarium* species can also cause allergic diseases in immunocompetent (sinusitis) and immunocompromised (mycotoxicosis) individuals (Nucci and Anaissie, 2007).

Other infections in immunocompetent patients caused by *Fusarium* spp. are osteomyelitis (bone infection), pneumonia (lung infection), fungemia (blood infection by fungi or yeast), septic arthritis (joint inflammation followed by infection) or sinusitis (sinus infection) (Nucci and Anaissie, 2007). There are many species of *Fusarium* that have been identified (more than 50) but only a few cause diseases in humans. These are primarily *F. solani*, which is the most common (~50 % of cases), and *F. oxysporum* (~20 % of cases) (Nucci and Anaissie, 2007; Lortholary, Fernández-Ruiz, & Perfect.,

2016). *F. falciforme* (one of the species belonging to *F. solani* complex) is an emerging and one of the most virulent *Fusarium* spp. associated with fusariosis and keratitis (Al-Hatmi et al., 2016). More than 300 cases of *Fusarium* keratitis were associated with contact lens cleaning solution causing outbreak in 2005-2007, where *F. solani* was predominant (Al-Hatmi et al., 2016). Individuals who are receiving corticosteroid treatment or are neutropenic are particularly at risk of infections. As with the *Mucormycetes* and *Scedosporium*, *Fusarium* causes localized infections in immunocompetent people as well as disseminated infections in immunocompromised hosts. Nucci and Anaissie, (2002) have described fusarial skin infections in both immunocompetent and immunocompromised patients based on MEDLINE report cases research between January 1966 and October 2001. Comparing to other fungi like *Candida* or *Aspergillus* species, infections caused by *Fusarium* represent >50 % of skin infections, while no other species accounts for more than 10 % (Nucci and Anaissie, 2002). Epidemiologically, *Fusarium* has been recovered from hospital air, general water systems and other environments (Nucci and Anaissie, 2007).

The number of invasive fungal infections has increased over the last two decades in severely ill, hospitalised patients, in which fusariosis (infection caused by *Fusarium* spp.) is a main skin or superficial infection (Scheel et al., 2013).

Virulence traits. *Fusarium* spp. have ability to produce mycotoxins, including trichothecenes, which are known to suppress humoral and cellular immunity and ability to damage host tissue (Jain et al., 2011). The ability to adhere to prosthetic material and production of proteases and collagenases is another virulent trait proposed by Jain et al. (2011). Factors important for *Fusarium* infections differ between plant and animal hosts. For instance, Nucci & Anaissie. (2007) showed that a mitogen-activated protein

kinase was virulent in tomato plant but not in mice in terms of *F. oxysporum* infection, whereas a pH response transcription factor was important for infection in mice but not in plants (Nucci & Anaissie., 2007). *Fusarium* spp. sporulates *in vivo*, facilitating hematogenous dissemination, leading to fungemia that occurs in 70 % cases in hematopoietic transplant recipients (Kadri et al., 2015).

1.4 Current antifungal treatments

As mentioned previously, the high morbidity and mortality rate due to fungal infections is very often due to a delayed diagnosis, misdiagnosis or underlying disease. But limited antifungal availability, high toxicity and species-specific antifungal resistance are also major problems. Below I will describe types of current antifungals and their mechanism of action.

Polyenes

Drugs such as amphotericin B or nystatin are polyenes and therefore target ergosterol, a sterol responsible for membrane fluidity and permeability in fungi (Scorzoni et al., 2017). Their mechanism of action is via binding ergosterol, disturbing membrane by forming a pore complex that is followed by leakage of important ions and cytoplasmic contents. Ergosterol of fungi has cylindrical three-dimensional structure, while cholesterol, sterol of mammalian membrane, sigmoid shape (Odds et al., 2003). Conformational difference between those two might explain better binding of ampB to ergosterol over cholesterol, but also be a reason for high toxicity of ampB to mammalian cells (Odds et al., 2003). However, the mechanism of action of amphotericin B is fairly controversial. Some researchers showed that disturbing the fungal membrane might not be necessarily linked to cell death. One alternative mechanism could be that amphotericin B influences auto-oxidation and formation of

free-radicals that causes cell death (Lamy-Freund et al., 1985). As also described by Scorzoni et al., 2017, who showed that polyenes are involved in a cascade of oxidation reactions with lipoproteins.

Amphotericin B is a natural product of *Sterptomyces nodus* (Lestner et al., 2013).

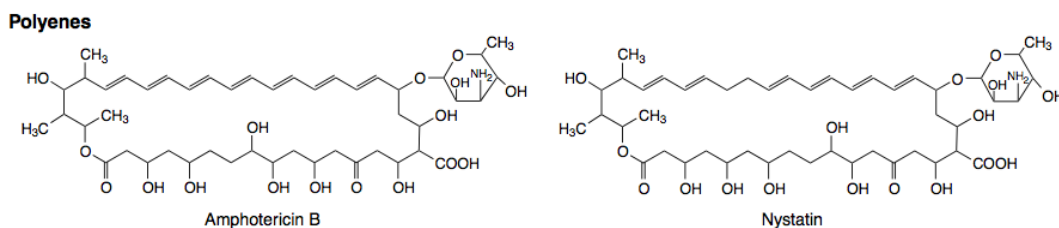


Figure 1.2 Chemical structure of polyene. Figure adapted from (Odds et al., 2003).

Azole

Another group of antifungals that target ergosterol are the azoles. Ergosterol depletion and replacement with usual sterols leads to disruption of membrane permeability and fluidity (Odds et al., 2003). Molecular target of azole is cytochrome P450 (Erg11 in yeast or Cyp51A in moulds), which catalyses the oxidative removal of the 14 α -methyl group from lanosterol in fungi (Odds et al., 2003; Cowen et al., 2014). Antifungal azoles bind to the ferric iron moiety (of the heme-binding site) located on the active site of this protein and blocks the enzyme's natural substrate lanosterol, disturbing the biosynthesis pathway (Cowen et al., 2014). P450, conformation of active site, differ between fungal species. Therefore, interaction between P450 and azole molecule might be different, and the inhibitory effect of azole within fungal species (Odds et al., 2003). To groups of azoles belong in between: imidazole (ketoconazole), triazoles (fluconazole, posaconazole, voriconazole, isavuconazole) and thiazoles (abafungin).

Systemically active azoles

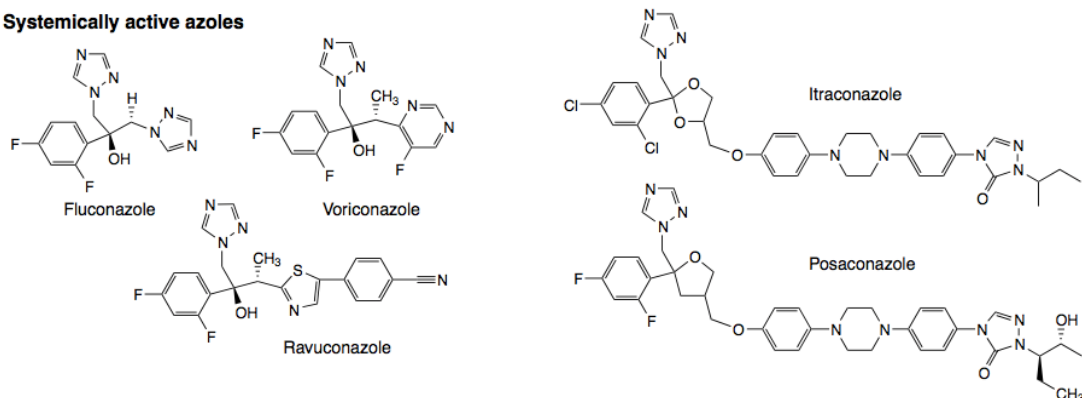


Figure 1.3 Chemical structure of azoles. Figure adapted from (Odds et al., 2003).

Echinocandin

Echinocandins are a class of drugs that includes antifungals such as caspofungin, micafungin and anidulafungin. Echinocandins target the cell wall, in particular proteins responsible for synthesis of β -1,3 glucans. This happens by blocking the enzyme glucan synthase, resulting in decreasing incorporation of glucose monomers between β -1,3 and β -1,6. This results in weakening the cell wall and fungal death (Scorzoni et al., 2017). As the cell wall composition varies between different fungal species, the echinocandin is not antifungal active against all fungal pathogens (Eschenauer et al., 2007).

Echinocandins

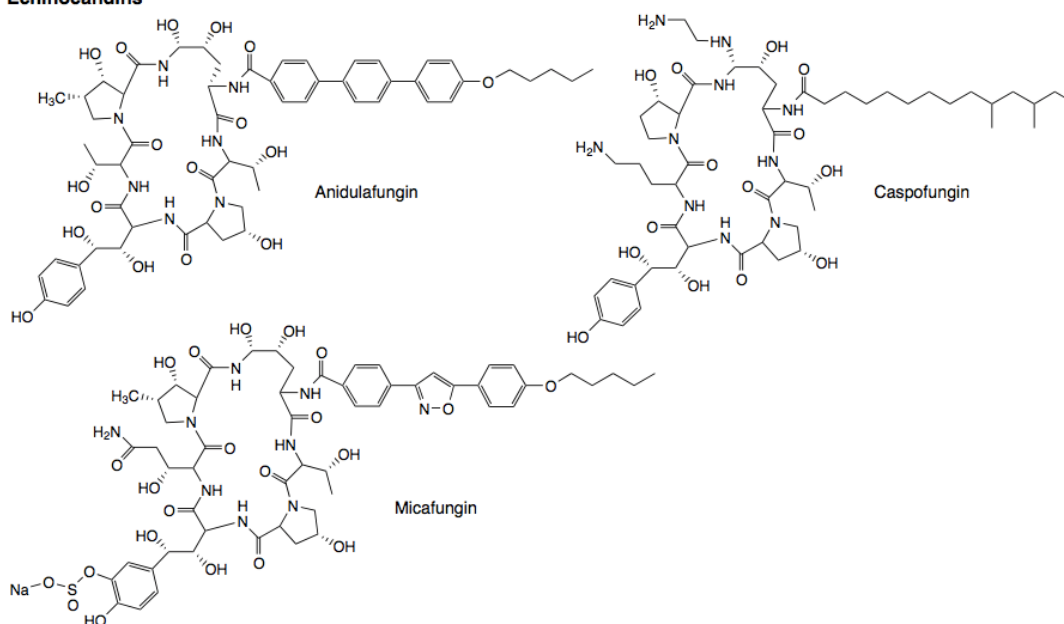


Figure 1.4 Chemical structure of echinocandins. Figure adapted from (Odds et al., 2003).

Alternative drugs under investigation

There are also alternative drugs being tested/proposed against filamentous fungi such as orotomides against *Aspergillus* or *Lomentospora* spp. (Hope et al., 2017); sphingolipids (Rollin-Pinheiro et al., 2016); *N*- Chlorotaurine against *Scedosporium*, *Lomentospora* spp. (Lackner et al., 2015) or proposing cytochrome P450 as a new drug target (Jawallapersand et al., 2014). Current treatment also includes hyperbaric oxygen and granulocyte colony stimulating factor (Rogers, 2008; Goldman et al., 2016). Galvez et al. (2017) isavuconazium sulfate as a treatment for burn infection of patients infected with mucormycosis.

1.4.1 Antifungal treatments for mucormycosis, scedosporiosis and fusariosis.

Mucormycosis. Effective treatment of mucormycosis requires an early diagnosis as well as prompt initiation of therapy. Treatment for mucormycosis include polyenes, except for *Cunninghamella* spp. and only two triazoles: posaconazole and

isavuconazole (but MIC value range is large for the second one) (Lortholary, Fernández-Ruiz & Perfect, 2016). Recommendation of antifungals used for treatment of mucormycosis include amphotericin B (ampB) and posaconazole (PSC). AmpB, has been a mainstay treatment for several decades despite its association with considerable kidney toxicity (Almyroudis et al., 2007). More recent lipid formulations of ampB (Ambiosome) are much less nephrotoxic and can be safely administered at higher doses for a longer period of time (Spellberg et al., 2009). PSC appears to be well tolerated, and is recommended to be used for treatment if there is ampB intolerance or in patients who did not respond to previous antifungal therapy (Enoch et al., 2011, Kim and Williams, 2014). A number of research groups have been testing different antifungals and their MICs against various *Mucormycetes* species and isolates and confirmed ampB as a first choice of treatment, followed by posaconazole (Spellberg et al., 2009; Rogers, 2008; Cornely et al., 2009; Enoch et al., 2011; Greenberg et al., 2006; Kim and Williams, 2014). Rogers (2008) suggested that PSC is a good oral treatment for mucormycosis and suggested that comparison of posaconazole and lipid amphotericin B should be tested as a primary treatment to find out which may have greater efficiency, although this has not yet been undertaken. Fluconazole, voriconazole, caspofungin and echinocandin showed poor or no activity against *Mucormycetes* spp. (Almyroudis et al., 2007). In addition, surgical debridement is an effective way to treat patients suffering from mucormycosis, although in practice there are difficulties in achieving this as important structures are often adjacent to necrotic tissues: particularly in rhino-orbital, sinu-nasal and especially, cerebral involvement (Cornely, Vehreschild, and Rüping, 2009). Therefore, there is a need for

improved antifungal treatment to reduce the need for such surgical (and often debilitating) treatments.

Scedosporiosis. As described above, scedosporiosis is common in both immunocompetent and immunocompromised patients; moreover, many isolates are highly resistant to antifungals, especially *L. prolificans*, which is multidrug resistant (Pellon et al., 2018 b).

Scedosporium, *Lomentospora* species have varying MIC (Minimum Inhibition Concentration)/MEC (Minimum Effective Concentration) distributions; therefore predicting susceptibility to any antifungal in a particular strain is problematic (Lackner et al., 2012). Lackner et al. (2012) have shown that *L. prolificans* is resistant to amphotericin B, intraconazole, posaconazole and isavuconazole (Lackner et al., 2012 and Zeng et al., 2004) and that these drugs show very limited efficacy towards *S. apiospermum*. Voriconazole has been shown to have an antifungal activity against *Scedosporium* spp. (Tucker et al., 2015). These results have been confirmed by Mello et al. (2016) showing only voriconazole being effective against *S. apiospermum* and *L. prolificans*, in comparison to ampB, caspofungin, fluconazole and intraconazole, all being non-effective against those strains. Similar observation were published by Gil-Lamaignere et al., 2002; Masukane et al., 2017; Wang et al., 2015. Posaconazole has also shown to have a good activity against *Scedosporium* spp. (Sabatelli et al., 2006). However, Lackner et al. (2012) have observed species-specific cross-resistance between *Scedosporium* spp. to different azoles. Recently Ramirez-Garcia al. (2018) in their review summarised that *Scedosporium* spp. are resistant to 5-flucytosine (drug targeting synthesis of fungal RNA and DNA), ampB, first generation triazole drugs, fluconazole and itraconazole. They are less susceptible to echinocandins, specifically

to caspofungin and anidulafungin, and have also shown resistance to the most recent triazole drug such as isavuconazole (Ramirez-Garcia al., 2018). For *Scedosporium/Lomentospora* spp. infections, European guidelines recommend voriconazole as a first line treatment and surgical debridement (Ramirez-Garcia al., 2018).

Therefore, investigating innate immune interaction with these pathogens is very important to understand difficulty with current antifungal resistance and development of new drugs.

Fusariosis. Invasive fusariosis contributes to 1/4 of cases of invasive fungal infections (Lortholary, Fernández-Ruiz, & Perfect., 2016). The current antifungal therapy of choice for fusariosis is amphotericin B, or/and voriconazole and posaconazole (Tortorano et al., 2008). However, these treatments show poor *in-vitro* activity (Pujol et al. 1997), and fusariosis involving haematogenous dissemination almost always results in mortality (O'Donnell et al., 2008). Therefore, clinical treatment of fusariosis essentially depends on the immune status of the host and the fungal route of entry (Tortorano et al., 2008). From a single cancer center with 44 cases of proven invasive fusariosis, 1/3 of patients had a breakthrough infection, despite the fact that most of them have been receiving prophylaxis treatment with voriconazole or posaconazole (Lortholary, Fernández-Ruiz, & Perfect., 2016). Al-Hatmi et al. (2016) investigated drug susceptibility among different strains of *Fusarium* and found them to be resistant to almost all antifungals (azoles and polyenes) being currently in use. While, Tortorano et al. (2008) showed ampB being the most effective against *Fusarium* spp., the distribution and pattern along different species and susceptibility to drugs is very variable.

Again, the host innate immune system plays a huge role in fighting against fungal infections. Schäfer et al. (2014) demonstrated that murine macrophages migrate towards and internalize germinated microconidia. Similarly, other research group has also shown importance of innate immune cells in fighting *Fusarium* spp. (Sun et al., 2010; Tarabishy et al., 2008; Jin et al., 2007; Karthikeyan et al., 2011).

1.4.2 Antifungal resistance

1,350,000 deaths are caused by fungal infections annually, with more than 300 million people suffering from it (Brown et al., 2012). Antifungal resistance is a rising problem in clinical cases and those might be due to few factors described below.

Widespread of antifungal agents in agriculture is one of cause of rising antifungal resistance; medical and surgical devices, such as catheters and artificial heart valves are known to be contributing to biofilm formation, therefore accompanying drug resistance (Cowen et al., 2014). Microbial resistance can be primary or secondary. Primary resistance occurs when the pathogen is inherently less susceptible to a given antifungal drug, while secondary resistance involves a pathogen acquiring resistance during antifungal treatment. Therefore, drug penetration to the site of infection may also contribute to antifungal resistance (subclinical reservoirs of fungal spores/cell that has not been reached/killed by drug are seeding new infection) (Cowen et al., 2014). Host immune status is one of most important factor in antifungal resistance as a fungistatic drug must work synergistically to control and clear the fungal abundance (Cowen et al., 2014).

Antifungal resistance mechanism is a broad and complex subject, therefore only a few mechanisms will be pointed out in this research thesis basing on the review of Cowen et al. (2014).

Azole resistance. Amino acid substitution in the drug target are common azole resistance mechanism known in fungal species; ERG11 mutations; efflux pumps, which recognize diverse chemicals enabling multidrug resistance; ABC proteins (ATP-dependent transporters such as CgCdr1, CgCdr2, CgSnq2, Afr1); MFS transporters (use the electrochemical proton-motive force to power drug efflux); multiple genomic alterations (e.g. loss of heterozygosity); mitochondrial dysfunction; biofilms formation and cellular stress response (Hsp90) (Cowen et al., 2014; Odds, Brown, & Gow, 2003; Robbins, Leach& Cowen, 2012).

Echinocandin resistance. This is commonly associated with amino acid substitutions in the Fks subunit of glucan synthase; multidrug transporters; biofilm growth; stress adaptation responses and fungal stress tolerance (Hsp90, cell wall integrity, protein kinase C, Ca^{2b}/calcineurin/Crz1, and high osmolarity glycerol cascades contributes to fungal cells survival following echinocandins treatment) and increased chitin synthesis (Cowen et al., 2014).

Antifungal resistance in *Mucormycetes* spp. *Mucormycetes* are resistant to triazoles (Gamaletsou, Walsh & Sipsas, 2018). Caramalho et al. (2017) research have shown that *Mucormycetes* spp. show *in vitro* resistance to the short-tailed azoles TDM (therapeutic drug monitoring) (median MIC >16.00 mg/l) and fluconazole (median MIC >64.00 mg/L) and variable susceptibility of those fungal species to long-tailed triazole posaconazole (MIC 2.00-8.00 mg/L) and the polyene amphotericin B (MIC 0.50-2.00 mg/L). Amino acid substitution in *Mucormycetes* spp. LDM (inhibition of lanosterol 14 α -demethylase) that differently affect the binding of short- and long-tailed may be the reason for this phenomenon (Caramalho et al., 2017). Similarly to those, Sagatova et al. (2016) have shown that *Mucormycetes* spp. are resistant to voriconazole as they

had substitution of Y129F, V and A of the active site of LDM F5, but not LDM F1. However, both LDM isoforms are expressed while exposed to voriconazole, they proposed those substitutions as a selective resistance mechanism of short-tailed azoles (Sagatova et al., 2016). Müller et al. (2018) research of azole activity in *Mucormyces* spp. have shown that their sterol composition has been alerted following azole treatment but did not lead to reduction of total sterol content. One of the substrates of the azoles target, enzyme C14-demythelase (eburicol), and its high amount might cause less accumulation of non-physiological intermediates due to azole treatment and its lower activity in comparison to other moulds (Müller et al., 2018).

Antifungal resistance in *Fusarium* spp. *Fusarium* spp. are intrinsically resistant to azole antifungals (Al-Hatmi, Meis and Sybren de Hoog., 2016). Azoles are cheap drugs that have a broad-spectrum activity and stability and are widely used in agriculture. Five azoles, such as propiconazole, bromuconazole, epoxiconazole, difenoconazole and tebuconazole are used for crop protection, however; they have been shown to be associated with function of CYP51 (gene encoding sterol 14 α -demethylase) and its role in antifungal resistance (Al-Hatmi, Meis & Sybren de Hoog., 2016). Mutations of *Fks1* gene might also contribute to the echinocandin resistance in *Fusarium* spp. (Katiyar & Edlind., 2009). Katiyar & Edlind. (2009) showed that spot 1 substitution P647A and F639Y in *Fks1* contributes to antifungal resistance in *F. solani*. Berthiller et al. (2013) have shown that efflux mechanism is also involved in antifungal resistance in *Fusarium* spp. The resistance mechanism is involved in effective efflux apparatus that is able to remove xenobiotics from its cells, which may reduce azole sensitivity (Berthiller et al., 2013).

Antifungal resistance in *Scedosporium/Lomentospora* spp. Antifungal resistance in *Scedosporium/Lomentospora* spp. is poorly understood to date. This might be due to the fact that, for example the *L. prolificans*, genome is not publicly available, so it limits any attempts of comparing the sequence to find homologues or differences with other, better studied, fungal species. Also, lack of genomic information restrict research from performing genetic-engineering experiments that would help to investigate antifungal resistant mechanism (Pellon et al., 2018 b). The few investigations to date are described below.

Research on *Scedosporium* and *L. prolificans* spp. have shown that mutations in *Fks1* gene, which is known from encoding catalytic subunit of the β -1,3-glucan synthase, is the factor for antifungal resistance for echinocandins (Ramirez-Garcia et al., 2018). Pellon et al. (2018 a, b) research propose molecular mechanisms that is related to antifungal resistance, and these are mutations on specific genes, such as *Fks* or *Erg11/Cyp51* as well as cellular and molecular changes developed by *L. prolificans* followed exposure to voriconazole, which developed resistance to this drug (Pellon et al., 2018 a, b). Johnson, Katiyar & Edlind (2011) have investigated acquired resistance or reduced echinocandin susceptibility (RES) association with two “hot spot” regions of *Fks1* and *Fks2* (β -1,3-glucan synthase). They have presented an evidence for W695F substitutions within hot spot 3 of both, *S. apiospermum* and *L. prolificans*, however, this single mutation did not fully accounted for RES profile in *Scedosporium* spp. suggesting that substitutions elsewhere within *Fks1* together with other factors might play a role in resistance (Johnson, Katiyar & Edlind., 2011). Biofilm formation ability by *Scedosporium* and *Lomentospora* spp. reported by Mello et al. (2016) might also contribute to antifungal resistance. Ramirez-Garcia et al. (2018) in their overview

of *Scedosporium/Lomentospora* spp. mention Japanese company, Eisai Co., who has discovered E1210 (first-in-class antifungal drug) that has an activity against fungal infections such as candidiasis, fusariosis and aspergillosis. Such a drug targets the inositol acylation step in the biosynthesis pathway of the glycosyl phosphatidyl inositol (GPI) anchor (Ramirez-Garcia et al., 2018). The mechanism of action of E1210 is to block the inositol acetylation step to cause defect in fungal cell wall biosynthesis, hyphal growth and ability of fungal cells adherence to biological substrates. This promising drug however, has been shown to have a 10-fold lower MIC against *S. apiospermum*, *S. aurantiacum* and *L. prolificans* than to voriconazole, which suggests those species developed a resistant mechanism against it which is currently unknown (Ramirez-Garcia et al., 2018).

1.5 Innate antifungal immunity

The innate immune system is the first line of defence against potentially fatal fungal infections. Macrophages and neutrophils (innate immune cells) are key phagocytes that mediate fungal killing (Erwig and Gow., 2016). Macrophages, a long-lived monocyte-derived cells, are patrolling the tissue and epithelia to target microbial prey by chemotaxis. In comparison, neutrophils are short-lived, abundant and rapid responders of the innate immune system (Erwig and Gow., 2016). Another important phagocyte are dendritic cells, whose interaction with fungal cells leads to activation of adaptive immune system (Erwig and Gow., 2016). Phagocytic clearance of fungal pathogens depends on ingestion of invading fungi through recognition of surface structures that comprise the fungal cell wall. Those structures are called pathogen-associated molecular patterns (PAMPs) and are recognised by structures on the surface of innate immune cells called pattern recognition receptors (PRRs). Fungal cell

wall is typically arranged in layers, such as chitin (the innermost layer); an *N*-acetylglucosamine polymer; and external layer consisting of β -1,3- and β -1,6-glucans (Lionakis, Iliev & Hohl., 2017). Many fungal species also employ an α -glucan layer (*H. capsulatum*); proteinaceous hydrophobin layer (*A. fumigatus*); *N*- O-linked mannans (*C. albicans*), glucuronoxylomannan, galctomannan capsule (*C. neoformans*) and melanin (*A. fumigatus*). Different layers of fungal cell wall are essential to immune detection as they consist of different PAMPs. Those are recognised by PRRs such as: C-type lectin receptors (CLRs), Toll-like lectin receptors (TLRs), nucleotide binding and oligomerization domain (NOD)-like receptors (NLRs) (Salazar & Brown., 2018).

CLRs can recognise PAMPs such as:

Dectin-1 (a β -glucan receptor; which is known to be involved in the recruitment of the Syk kinase through immunoreceptor tyrosine-based activation motif (ITAM)-containing cytoplasmic domain that is phosphorylated by a Src family kinase). Dectin-1 can, in between, induce phagocytosis, respiratory burst, chemokine and cytokine production, and negatively regulate fungal-mediated neutrophil extracellular trap (NET) formation (Salazar & Brown., 2018).

Dectin-2 cluster (clec6a = dectin-2, clec4d = MLC and clec4e = macrophage-inducible C-type lectin or Mincle) recognises α -mannans and O-lined mannoproteins (Salazar & Brown., 2018). Those receptors drive intracellular signalling via Syk-CARD8 pathway. Its interaction with fungi can cause cytokine and ROS production and fungal killing.

DC-specific intracellular adhesion molecule-3-grabing nonintegrin (DC-SIGN) recognises mannose-containing fungi and modulates signalling pathways, including signals induced from TLRs, therefore can induce adaptive immune responses depending on the nature of the pathogen (Salazar & Brown., 2018).

Mannose Receptor (MR) recognises carbohydrates terminated in D-mannose, L-fucose or N-acetyl glucosamine. MR are involved in phagocytosis, induce IL-17, increasing IL-10 and reducing pro-inflammatory cytokine production (Salazar & Brown., 2018). Additionally, there are other mannose-binding lectins such as galectins and mannose-binding lectin (MBL).

Toll-like receptors are inducing signalling pathway through adaptor proteins ((myeloid differentiation primary response 88 (MyD88); TIR domain-containing adapter-inducer interferon- β - (TRIF)). TLR4 is known to recognise O-linked mannosyl residues and rhamnomannans inducing TNF- α and IL-6 by macrophages; TLR2/TLR6 and TLR2/TLR1 are known to interact with phospholipomannan and glucuronoxylomannan to produce TNF- α and nitric oxide by macrophages (Salazar & Brown., 2018).

NLRs consist of subfamilies called NLRA, NLRB, NLRC and NLRP. NLRP3 is the best characterised, being involved in production of IL-1 β and IL-18. Several yeast cell wall preparations shown to be able to activate NLRP3 which is involved in phagocytosis and inflammation (Salazar & Brown., 2018).

Additional β -Glucan receptors are complement receptor3 (CR3). This receptor mediates fungal phagocytosis and killing via complement-dependent manner as well as drive Th1 and Th17 responses (Salazar & Brown., 2018).

Once fungal pathogens are ingested, the harsh environment of the phagosome is thought to kill and eradicate the pathogen. To do so, the phagosome undergoes extensive remodelling which depends on the membrane fusion and fission of endosomal compartments (Erwig and Gow., 2016). Following that, the phagosomal lumen becomes acidified (via action of vacuolar ATPase proton pumps) and by activating oxidative- (the respiratory burst, reactive nitrogen intermediates) and non-

oxidative mechanisms (antimicrobial peptides, hydrolases and lamination of nutrients) attempt to kill and destroy pathogen. This process is called phagosome maturation (Erwig and Gow., 2016). Above mentioned PAMPs define the contribution of PRRs involved in phagocytosis, downstream signalling pathway that mediates RAB function (RAB GTPases mediates the maturation of phagosome, a key proteins in this process) and outcome of the host-pathogen interaction (Erwig and Gow., 2016). Despite this beautiful and complex process, some fungal pathogens have developed a way to avoid phagosomal killing, by inhibiting phagosomal maturation, avoiding degradative environment or via escaping from the phagosome (Erwig and Gow., 2016). For instance, *Candida albicans* can neutralise the acidic pH of macrophage phagosome by extrusion of ammonia, similarly to *Helicobacter pylori*, which is able to produce ammonium from urea to buffer the phagosomal pH; *C. albicans* manipulates RAB GTPs (RAB14) to increase its survival within phagosome; *C. neoformans* is able to escape from phagosome via non-lytic expulsion “vomocytosis”, while *A. fumigatus* via cell-to-cell transfer “lateral transfer”; *Histoplasma capsulatum* inhibits accumulation of V-ATPase; (Flannagan et al., 2012; Erwig and Gow., 2016; Shah et al., 2016; Gilbert et al., 2017).

1.5.1 Host-pathogen interaction of innate immune cells with *Mucormycetes*, *Scedosporium/Lomentospora* and *Fusarium* spp.

The host-pathogen interaction with emerging fungal pathogens such as *Mucormycetes*, *Scedosporium/Lomentospora* and *Fusarium* spp. are very understudied and the resources of literature are still limited. However; some of the published studies are summarised below.

Macrophages

Studies of Kraibooj et al. (2014) on *Lichtheimia corymbifera*, belonging to *Mucormycetes* spp. have shown that different stages of spore activity (resting/dormant phase and swelling in size phase/increased metabolism) and opsonized spores, are better phagocytosed by macrophages in comparison to attenuated strain (strain that exhibited lower virulence) in the same conditions. They have also shown that opsonized spores of virulent strain are better phagocytosed in comparison to swollen and resting one; however opsonization had not effect on attenuated strain in comparison to swollen spores in terms of opsonization (Kraibooj et al., 2014). *Mucormycetes* spp. spores' germination can be effectively inhibited by healthy murine bronchoalveolar macrophages, however; they fail to kill spore of *R. oryzae*; whereas, rabbit bronchoalveolar macrophages fungicidal activity against this strain greatly depends on spore developmental stage (Waldorf., 1989; Levitz et al.,1986). A recent study of Andrianaki et al. (2018) showed that primary macrophages are able to completely inhibit *Mucormycetes* growth and are susceptible to oxidative and non-oxidative effector mechanisms of macrophages. Moreover, they showed that *Rhizopus* survival within macrophages is stimulated via melanin-induced phagosomal maturation arrest and iron homeostasis within macrophages impacts *Mucormycetes* survival within phagosome (Andrianaki et al., 2018). In comparison to *Mucormycetes* spp., macrophages have been shown not to be able to inhibit germination of ingested spores of *Fusarium* spp. Schafer et al. (2014) demonstrated that murine macrophages migrate towards and internalise germinated microconidia. They have also shown that survival of the macrophages that have engulfed germlings (germinated microconidia) depends on the number of engulfed fungal germlings (Schafer et al., 2014). 93.4% of

macrophages that internalized 4 or more germings of *F. oxysporum* were killed by the fungus (Schafer et al., 2014), due to the ability of the fungal hyphae to grow inside the phagosome, leading to their escape and host-cell lysis.

One of first phagocytic cells to approach *Scedosporium/Lomentospora* are macrophages, which engulf conidia to prevent conidial germination (Gil-Lamaignere et al., 2003). Gil-Lamaignere et al. (2003) have shown that the conidia of two isolates of *S. apiospermum* (SA54A- isolate from heart biopsy from fatal disseminated infection, and SA1216- isolate from leg skin from localized subcutaneous, treated infection) can be phagocytosed by monocyte-derived macrophages (MDMs). The phagocytosis by human polymorphonuclear leukocytes (PMNs) and mononuclear leukocytes (MNCs) of conidia of both isolates was the same as in MDMs, however there were differences in interaction and susceptibilities of these two isolates to the oxidative burst response. SA54A- isolate exhibited generated higher oxidative burst production without relative hyphal damage suggesting its high level of resistance to oxidative burst then SA1216- isolate.

Chamilos et al. (2008 a) used *Drosophila melanogaster* to show that treatment with dexamethasone (steroid) impaired phagocytic efficacy against *R. oryzae* (belonging to *Mucormycetes* spp.) in comparison to healthy flies.

Voelz, Gratacap & Wheeler., (2015) research using zebrafish larva have shown importance of macrophages in response to *Mucormycetes* spp. in comparison to zebrafish larvae depleted in macrophages which showed higher mortality rate.

Vacher et al. (2015) research have shown that spores of *Fusarium culmorum* stimulates secretion macrophage inflammatory protein (MIP)-1 α , MIP-1 β , MIP-2, monocyte chemoattractant protein (MCP)-1, RANTES, IL-12p40, TNF- α , IL-6, IL-

12p70, IL-33, G-CSF and interferon gamma-induced protein (IP-10). Overall, macrophages have responded to *F. culmorum* releasing all those inflammatory proteins but not to its secreted components (mycotoxins). This suggest potential stimulation of innate immune system by this pathogen.

Neutrophils

A recent review by Ghuman & Voelz (2017) summarises the importance of neutrophils in interaction with *Mucormycetes* spp. Neutrophils have been shown to have a fungicidal activity against *R. oryzae*. Its NP-1 and NP-2 cationic peptides have been shown to be active against resting *R. oryzae* spores but not against swollen spores (Waldorf., 1989).

Chamilos et al. (2008 b) have shown that *Rhizopus* hyphae induce expression of pro-inflammatory genes of neutrophils (*tnf-α* and *il-1b*). Similarly to *Mucormycetes* hyphae, *Fusarium* hyphae are also killed by neutrophils. Sun et al. (2010) showed that neutrophils are able to kill *Fusarium* via binding to fungal hyphae and secreting reactive oxygen species. They showed that TLR4 expression on neutrophils is necessary for recognition and killing of fusarial hyphae (Sun et al., 2010). Importantly, they have also shown that IL-1R1/MyD88 signalling regulated chemokine production and neutrophil recruitment during keratitis both trauma-induced and biofilm formation. Taylor et al. (2014) research have also shown involvement of neutrophils in *Fusarium* killing and release of IL-17 by those. Similarly, Tarabishy et al. (2008) showed that TLR4 is important for *Fusarium* spp. killing but not neutrophil recruitment.

Voelz, Gratacap & Wheeler., (2015) research using zebrafish larva have also shown the neutrophils being attracted to the site of *Mucormycetes* injection.

Dendritic cells (DCs)

Rhizopus spores do not activate DCs, in comparison, hyphae does stimulates release of IL-23 by DCs (Chamilos et al., 2010). They have shown that β -glucan cell wall component of *Rhizopus* hyphae is necessary for IL-23 production and T_H-17 responses by DCs. In comparison, spores of *Fusarium culmorum* has been recognised by DCs via Dectin-1 receptor, but not MyD88 and TRIF-dependent TLR (Vacher et al., 2015).

AIMS AND OBJECTIVES

The main aim of this project is to investigate novel treatments for trauma associated fungal pathogens. To achieve this aim, the host-pathogen interaction of such fungal pathogens was investigated in addition to assessing the effectiveness of alternative antifungal therapies.

In the following chapters I will test (Figure 5):

Acetic acid concentrations as a fungistatic and/or fungicidal treatment against trauma-associated fungal species such as *Mucormycetes*, *Scedosporium*, *Lomentospora*, *Fusarium*, *Candida* and *Cryptococcus* spp.

Blue light antifungal activity against *Mucormycetes*, *Scedosporium*, *Lomentospora*, *Fusarium* and *Candida* spp.

Scedosporium, *Lomentospora* spp. interaction with innate immune cells.

Ex-vivo skin model as a novel model to investigate pathogenicity of trauma associated fungal species.

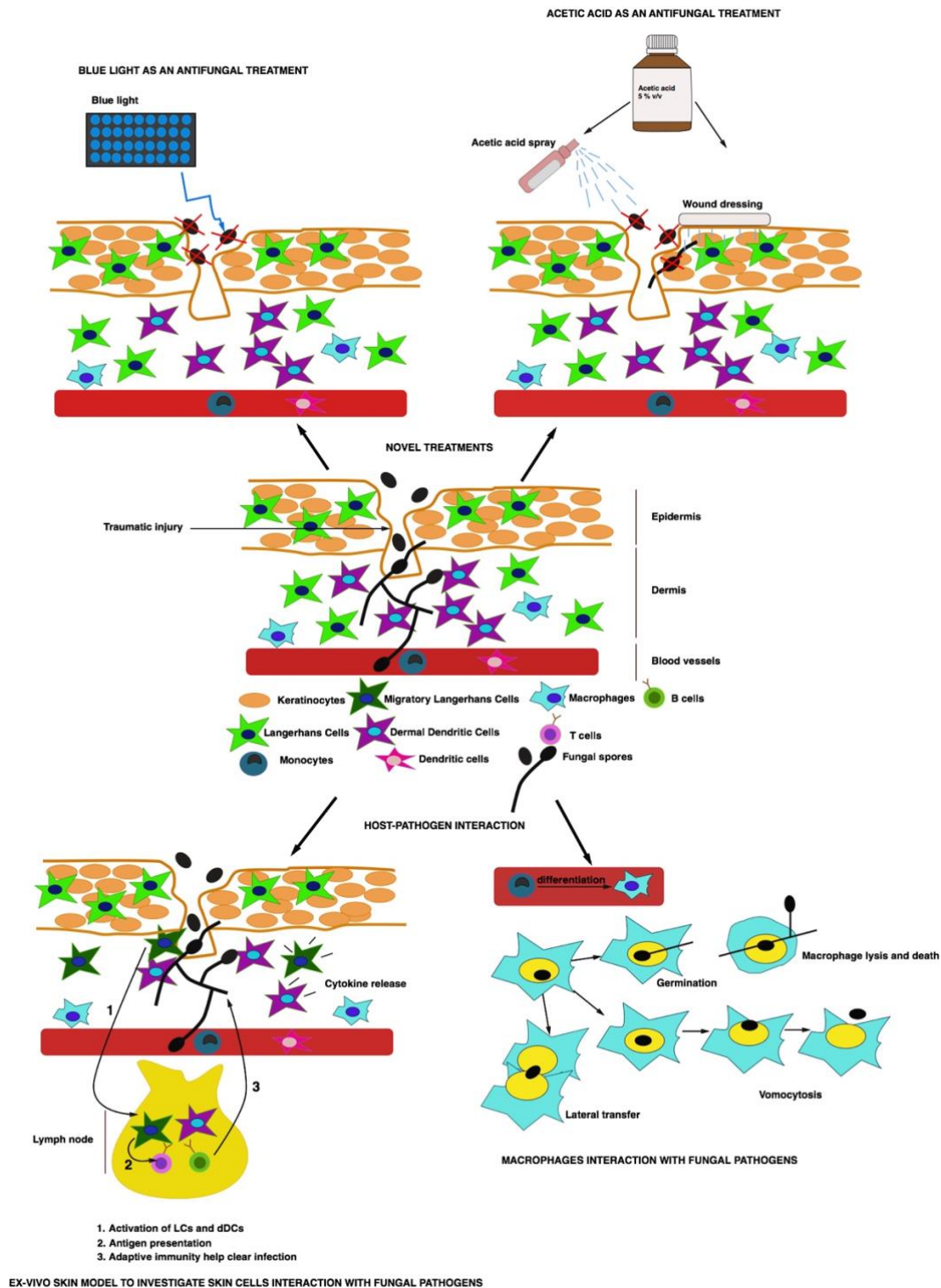


Figure 1.5 Visualisation of objectives to develop novel approaches to treat trauma-associated fungal infections.

2. ACETIC ACID AS AN ANTIFUNGAL TOPICAL TREATMENT FOR TRAUMATIC INJURY INFECTIONS.

Question

Is acetic acid, a well known antibacterial treatment, effective against various genera and species of fungi associated with traumatic injury? Can this treatment be used as a topical treatment for fungal disease?

The majority of the work presented in this chapter has been published in:

Trzaska, W.J. et al. (2015) pH manipulation as a novel strategy for treating mucormycosis. *Antimicrob Agents Chemother*, 59, 6968-6974.

2.1 Introduction

Acetic acid has been used for thousands of years as an antimicrobial agent. During the American Civil War and World War I, apple cider vinegar was used as a wound dressing, whilst during the great plagues of Europe, thieves used vinegar to avoid catching infection when stealing valuables from the dead bodies of plague victims (Fraise et al., 2013). More recently, it has been used to treat burn wound infections, and has shown activity against various bacterial pathogens (Ryssel et al., 2009; Nagoba et al., 2013). In addition, it is commonly used for eliminating bacteria from fresh products, or curing acute otitis externa (Cortesia et al., 2014). Ryssel et al. (2009) tested acetic acid activity against common bacterial strains found in burn patients and showed successful inhibition of multi-drug-resistant *P. aeruginosa* and *A. baumannii*. Acetic acid was also found to be bacteriostatic against multiple antibiotic resistant strains of *P. aeruginosa* at concentrations as low as 0.5-5 % (Nagoba et al., 2013). Levine and Fellers (1940) compared the efficacy of acetic, lactic and hydrochloric acid against *Salmonella typhimurium*, *Saccharomyces cerevisiae*, and *Aspergillus niger*, their research showed that acetic acid is more toxic to those organisms than lactic or hydrochloric acid. The mechanism of acetic acid action against bacteria, fungi and yeast remains unknown. Although historically attributed to pH (Kahlenberg and True, 1896) more recent work (Higgins and Brinkhaus, 1999) has stated that the mechanism of inhibition of microbial growth by organic acids is not considered to be a pH phenomenon. Cottier et al. (2015 a) investigated the transcriptional response of *C. albicans* to weak organic acids such as acetic, lactic, propionic and butyric acids. During the physiological response of *C. albicans* to weak acids, 16 genes were identified to play an important role (Cottier et al., 2015 a). Several

lines of research have shown that iron uptake has an important role of fungal response to weak acids (Mira et al., 2010; Cottier et al., 2015 a). Cottier et al., (2015 a) suggested RNA synthesis and ribosome biogenesis genes as a pathway regulated in response to such treatment. Since chronic exposure of *C. albicans* to weak organic acids down-regulated of RNA synthesis and ribosome genesis genes, significantly reducing total level of RNA. Cottier et al. (2015 b) also demonstrated a role for *MIG1*, a single gene, which confers weak acid resistance. This gene was previously known for its role in glucose repression, and now, Cottier et al. (2015 b), has confirmed its role in resistance to acetic, propionic, butyric and lactic acids.

The fungal species such as *Mucormycetes*, *Scedosporium*, *Fusarium* and *Candida* are commonly associated with traumatic injuries. Current topical treatment for wound infections is very expensive and often ineffective (Drosou et al., 2003). Therefore, there is an urge for developing novel treatments that are cheap, accessible and effective across different fungal species. Therefore, we have investigated here the antifungal activity of acetic acid and showed that fungal spores treated with acetic acid undergo rapid acidification of their cytoplasm and become arrested without germination. Thus, we propose that early application of dilute acetic acid may represent an effective and low-cost strategy to minimize trauma-associated fungal infections in traumatic wounds.

2.2 Materials and Methods

Unless otherwise stated all reagents were purchased from Sigma Aldrich UK.

Strains used in experiments. *Lichtheimia corymbifera* 9.6002134 and *Rhizopus microsporus* 12.6652333 (clinical isolates), *Mucor circinelloides* NRRL3631 and *Mucor circinelloides* CBS277.49 (Li, et al., 2011); *Scedosporium apiospermum* IHEM 14462 and *Scedosporium (Lomentospora) prolificans* IHEM5608; *Fusarium oxysporum* IHEM 25499 and *Fusarium solani* IHEM 6092; two isolates of *Cryptococcus neoformans*, H99 and KN99 (Nielsen et al., 2003); *C. albicans* SN152 (Noble and Johnson, 2005) and *C. albicans* DAY286 (Nobile and Mitchell, 2009). All *Mucormycetes* strains were grown on Sabouraud agar plates (VWR) for at least 10 days before use. All *Scedosporium*, *Lomentospora* and *Fusarium* strains were grown on Potato Glucose agar plates for at least 10 days before use. *Cryptococcus* spp. and *Candida* spp. were grown on YPD agar plates for 2 days, then grown over night in YPD broth, rotating at 25° C before use.

Media. Acetic acid experiments were performed in Sabouraud broth (Fisher Scientific) for *Mucormycetes*, *Scedosporium*, *Lomentospora* and *Fusarium* spp., and YPD broth for *Cryptococcus* and *Candida* spp. with different concentrations of acetic acid (Table 1). For hydrochloric and lactic acid treatments, the relevant pH in Sabouraud broth was matched with the pH generated by the different concentrations of acetic acid. Spores of *Mucormycetes*, *Scedosporium*, *Lomentospora* and *Fusarium* spp. were washed with PBS (Fisher Scientific), spun down and re-suspended in PBS for cell counting in a haemocytometer. Spore counts were adjusted to a final count of 1×10^4 cells per ml. *Candida* and *Cryptococcus* cells were grown over night in YPD broth prior to experiment, counted and adjusted to final count of 5×10^5 cells per ml.

Fungal growth inhibition assays for *Mucormycetes*. 5 % acetic acid (Tayside Pharmaceuticals, Dundee, UK) was serially diluted into Sabouraud media to produce concentrations of 2.5 %, 1.25 %, 0.63 %, 0.31 %, 0.16% and 0.08 %. The pH of each of these concentrations of acetic acid was measured and then pH-matched solutions of hydrochloric acid or lactic acid were produced in Sabouraud (Table 1). For pH-neutralised experiments, media were generated as above but then 1M NaHCO₃ was added to return the pH to that of the control. Fungal growth rate was measured by OD₆₀₀ reading at time 0 (time of spore inoculation to acetic acid), and after 24 and 48 hours using a microplate reader (BMG Labtech). Plates were incubated at 37° C in between reads, without shaking. To estimate spore killing, samples from the concentrations of acetic acid in which no growth was observed were plated onto Sabouraud agar plates, and the number of colony forming units (CFU) were counted 24 hours later. Each experiment was performed with at least three experimental replicates and three biological repeats.

Fungal growth inhibition assays for *Scedosporium*, *Lomentospora*, *Fusarium* and *Candida* spp. 5 % acetic acid was serially diluted into Sabouraud media for *Scedosporium*, *Lomentospora* and *Fusarium*, and in YPD broth for *Candida* to produce concentrations of 2.5 %, 1.25 %, 0.63 %, 0.31 %, 0.16 % and 0.08 %. And then 0.8 %, 0.4 %, 0.2 % and 0.1 % for *Scedosporium*, *Lomentospora* and *Fusarium* spp. Time-lapse imaging was performed on fungal spores using a Nikon Eclipse Ti microscope with ELWD 0.52NA 20x Ph1 objective for 18-20 hours, in 37° C, humidified with no CO₂. Movies were analysed and prepared using NIS Elements software. Each experiment was performed with at least two experimental replicates.

Fungal growth inhibition assays for *Cryptococcus*. To estimate spore killing, fungal cells were incubated in the concentrations of acetic acid of 5 %, 2.5 %, 1.25 %, 0.8 %, 0.6 %, 0.4 %, 0.3 %, 0.2 %, 0.16 %, 0.1 %, 0.08 % for 48 hours, then were plated onto YPD agar plates, and the number of colony forming units (CFU) were counted 24 hours later. Each experiment was performed with at least three experimental replicates.

Time lapse imaging for germinated *Mucormycetes*. Time-lapse imaging was performed on fungal spores either prior to, or shortly after germination, using a Nikon Eclipse Ti microscope with LWD 0.52 20x objective for 18 hours in 37° C, humidified with no CO₂. Movies were analysed and prepared using NIS Elements software.

Time of action for acetic acid killing of *Mucormycetes*. To test how rapidly acetic acid impacts on fungal viability, spores were incubated for 0, 15, 30, 60, 120, 180, 240 and 300 minutes in 2.5 % acetic acid in cRPMI (media to mimic blood environment) (complete RPMI media 1640 (cRPMI) - phenol free (GIBCO) + 100 U/ml of Penicillin and 100 U/ml of Streptomycin, 2 mM of L-glutamine and 10% FBS and then plated onto Sabouraud plates for subsequent colony counting 24 hours later. Scoring for colony forming units at this early time point requires more careful observation but avoids problems of extensive filamentation, leading to “fused” colonies.

Measurement of pH_i. To determine intracellular pH (pH_i). Fungal spores at a concentration of 6×10^6 /ml were incubated in RPMI medium at 37° C and 5 % CO₂ for 4 h before imaging. Cells were then incubated with 5 µM BCECF-AM [2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester; Life Technologies, USA] (which is used to estimate vacuolar and internal pH) for 1 h and resuspended in

fresh medium to allow full deesterification of the dye. Intracellular pH was measured via single-cell ratiometric imaging at x60 magnification using an Olympus IX81 (Olympus, United Kingdom) coupled to a monochromator-based illumination system (Cairn Research, United Kingdom) and an Evolve 512 EMCCD (Photometrics, USA) digital camera; image acquisition was controlled using MetaFluor (Molecular devices, USA) acquisition software. Fluorescence emission for excitation wavelengths centered at 490 nm and 436 nm was captured at 530 nm, and ratios were obtained for individual cells after background subtraction. Ratios were converted to intracellular pH values after *in situ* calibration as described by James-Kracke, 1992. Briefly, after the initial germination stage, cells were permeabilised in RPMI supplemented with 100 μ M nigericin (Thermofisher Scientific) and 150 mM KCl and subsequently exposed to extracellular pH ranging from 4.5 to 7.5 (acetic, lactic and hydrochloric acids). BCECF-AM (Thermofisher Scientific) ratios were then converted to intracellular pH (pH_i) values using the following equation:

$$pH_i = pK_a - \log \left[\frac{(R_{max} - R)}{R - R_{min}} \times \frac{F_{base436}}{F_{acid436}} \right]$$

Where pK_a is the acid dissociation constant for BCECF-AM, *R* is the ratio of the emission fluorescence signals measured at 530 nm when the fluorophore is excited at 490 nm and 436 nm, respectively, *R*_{max} is the ratio for maximum fluorescence measured at pH 7.5, *R*_{min} is the ratio for minimum fluorescence measured at pH 4.5, and *F*_{base436}/*F*_{acid436} is the ratio of fluorescence signals at 436 nm under the basic and acid conditions used to obtain *R*_{max} and *R*_{min}.

Statistical analysis. Statistical analysis was conducted using two-way ANOVA with Dunnett's multiple comparison test. *p < 0.05, **p < 0.01, ***p < 0.001.

2.3 Results

Acetic acid shows strong antifungal activity against *Mucormycetes* species.

Ubiquitous fungal spores are the infectious agent for mucormycosis. This disease depends on germination of fungal spores with subsequent tissue invasive hyphal growth. To test whether spore germination of *Mucormycetes* was inhibited by acetic acid, spores of *Rhizopus microsporus* 12.6652333, *Lichtheimia corymbifera* 9.6002134 and *Mucor circinelloides* NRRL3631 and CBS277.49 (Li et al., 2011), were inoculated into Sabouraud broth (to mimic nutrient-rich conditions) with varying acetic acid concentrations. *Mucormycetes* grow as filamentous fungi and thus OD measurement is problematic for monitoring growth rates, but is very successful in monitoring the onset of germination. Acetic acid impaired germination in all isolates at concentrations as low as 0.3 % (Figure 2.1 & 2.2). To test whether this effect was fungistatic or fungicidal, spores were plated onto Sabouraud agar plates and the number of colonies was counted after 24 hours. At concentrations of 2.5 % and above no viable colonies could be recovered (Figure 2.3). Time course analysis indicated that this fungicidal activity peaks following approximately four hours of exposure to 2.5 % acetic acid (Figure 2.4). Thus acetic acid strongly suppresses fungal germination at very low concentrations and is potentially fungicidal at concentrations above 2.5 %.

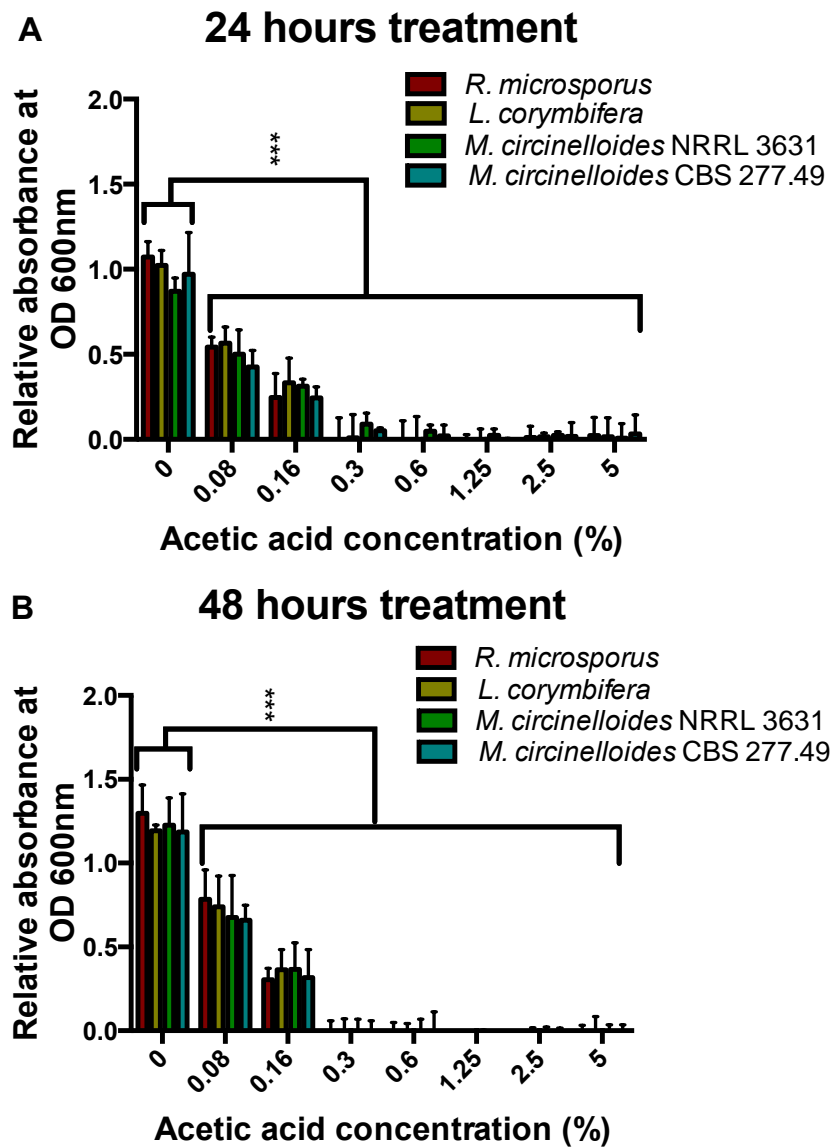


Figure 2.1 Acetic acid shows strong antifungal activity against several species of *Mucormycetes*.

Acetic acid inhibits fungal spore germination. Spores were grown in sabouraud media and growth assessed by OD₆₀₀ nm measurements. Graphs show OD measurement after 24 (A) and 48 (B) hours. Error bars represent standard deviation (n=4, three experimental replicates at each time). Statistical analysis was conducted using two-way ANOVA with Dunnett's multiple comparison test. *p < 0.05, **p < 0.01, ***p < 0.001.

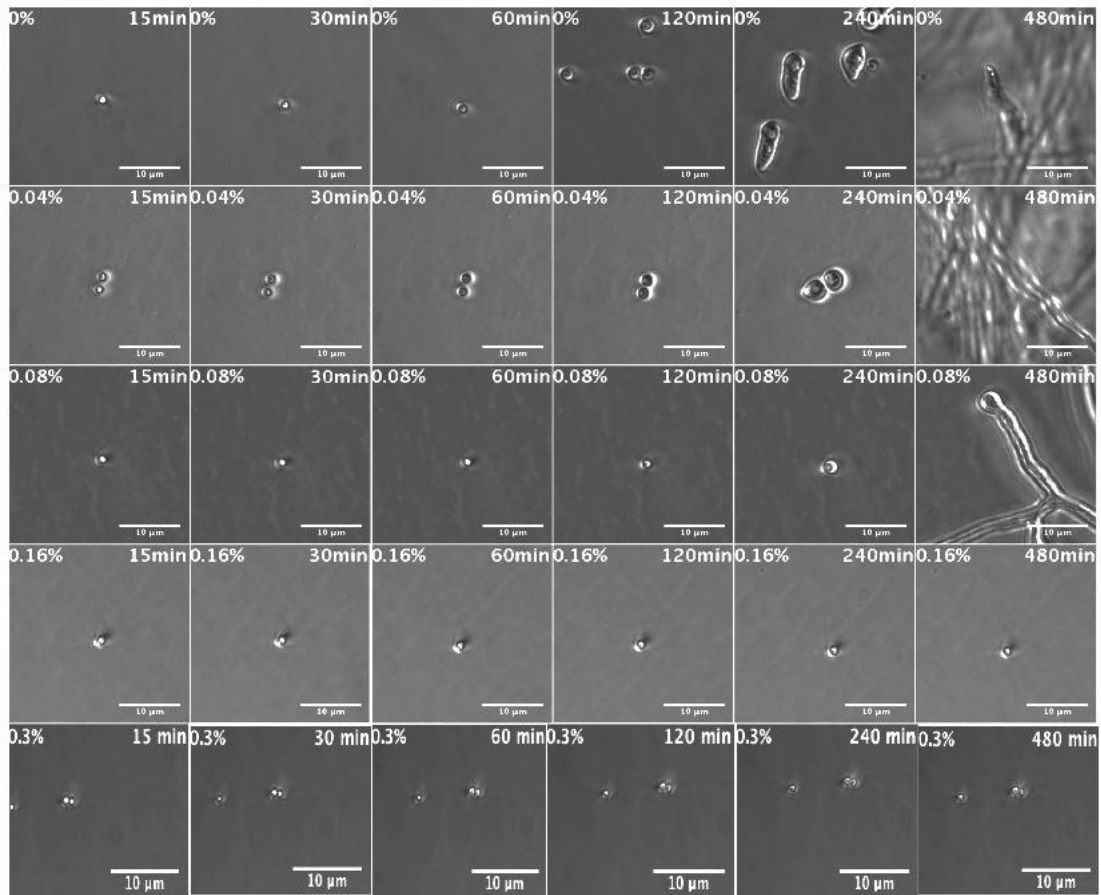
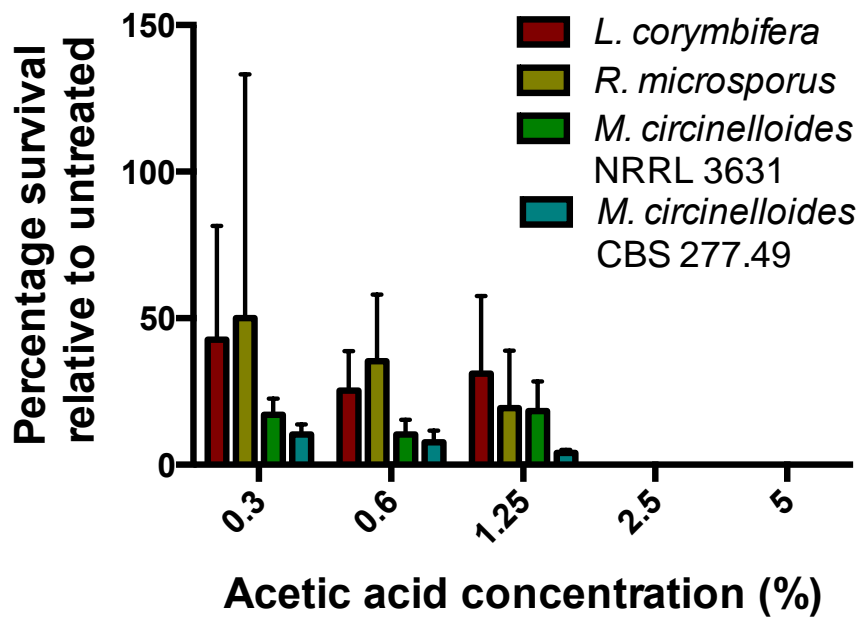


Figure 2.2 Visualization of acetic acid activity against *R. microsporus*.

Time-lapse imaging shows acetic acid concentrations of 0.16 % strongly inhibit germination. Acetic acid percentage (%) is presented in the top left corner of each image.

A 24 hours treatment



B 48 hours treatment

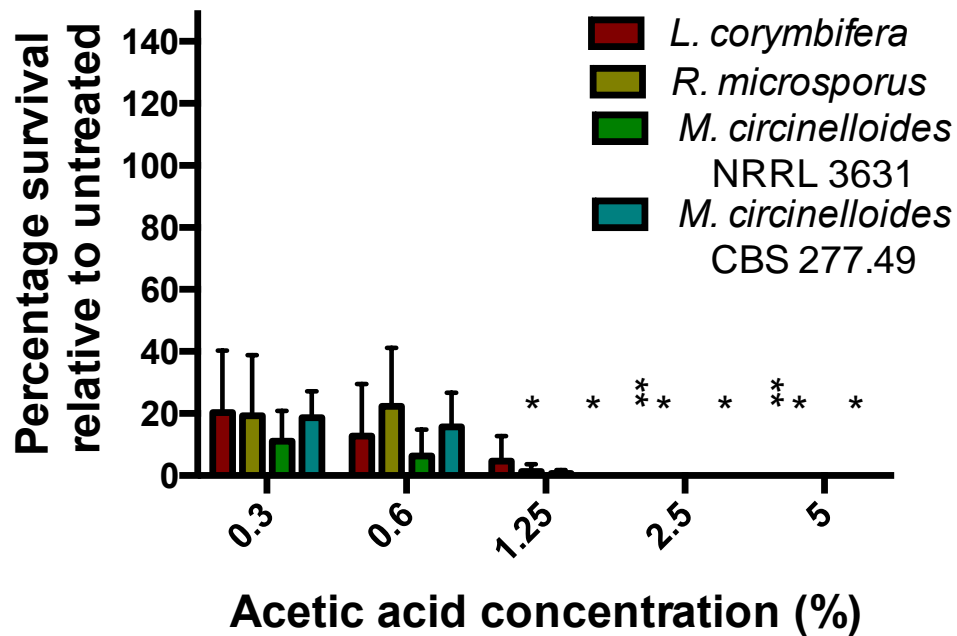


Figure 2.3 High concentrations of acetic acid are fungicidal.

Acetic acid is fungicidal to spore at concentrations of 2.5 % and above. Spores were grown in Sabouraud media supplemented with different concentrations of acetic acid for 24 and 48 hours and then plated for colony-forming unit (CFU) counts. Graphs show percentage survival related to untreated. Error bars represent standard deviation (n=3, three technical replicates at each time) and statistical analysis was conducted using two-way ANOVA with Dunnett's multiple comparison test in which spore survival in different concentrations of acetic acid for each strain was compared to 0.3 % treatment. *p < 0.05, **p < 0.01, ***p < 0.001. There was no 0 % (control samples) included in this study as an experimental error.

Time of action

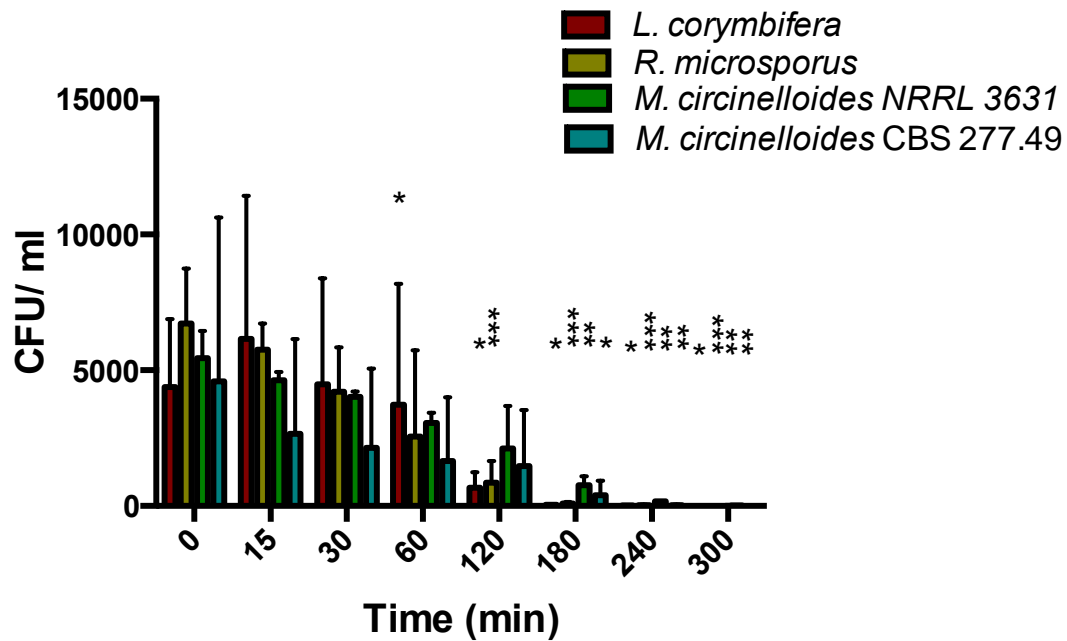


Figure 2.4 Time course showing activity of 2.5 % of acetic acid.

The fungicidal activity of 2.5 % acetic acid requires prolonged incubation. Spores were grown in RPMI media supplemented with 2.5 % acetic acid and plated onto sabouraud agar plates every 15, 30, 60, 120, 180, 240 and 300 minutes. The number of colonies was counted 24 hours later. Error bars represent standard deviation (n=3, three experimental replicates at each time) and statistical analysis was conducted using two-way ANOVA with Dunnett's multiple comparison test (compared to 0 min). *p < 0.05, **p < 0.01, ***p < 0.001.

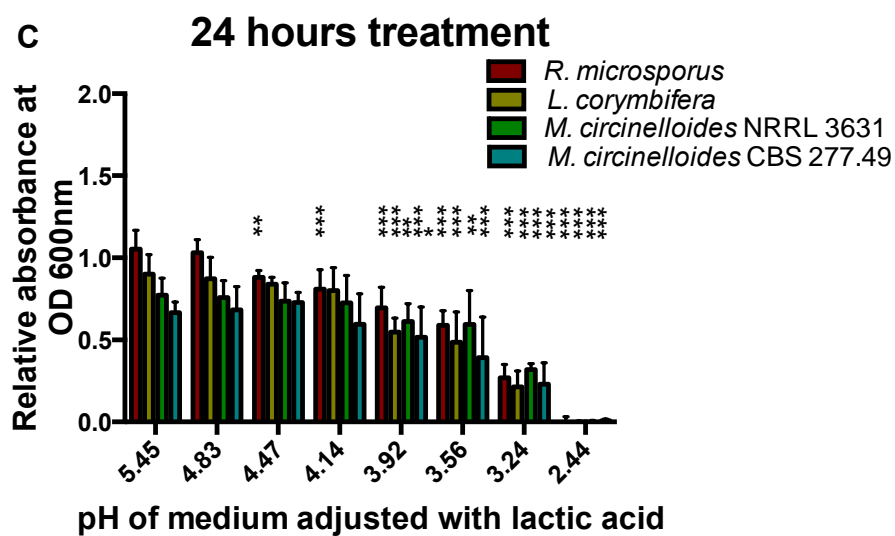
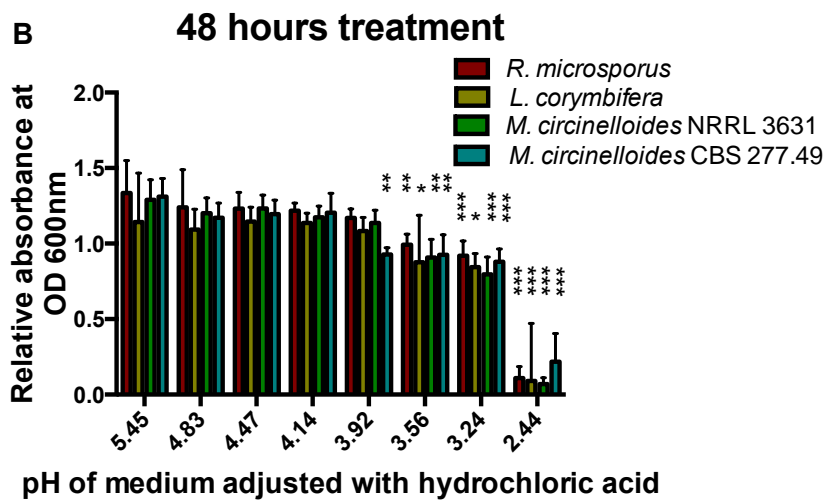
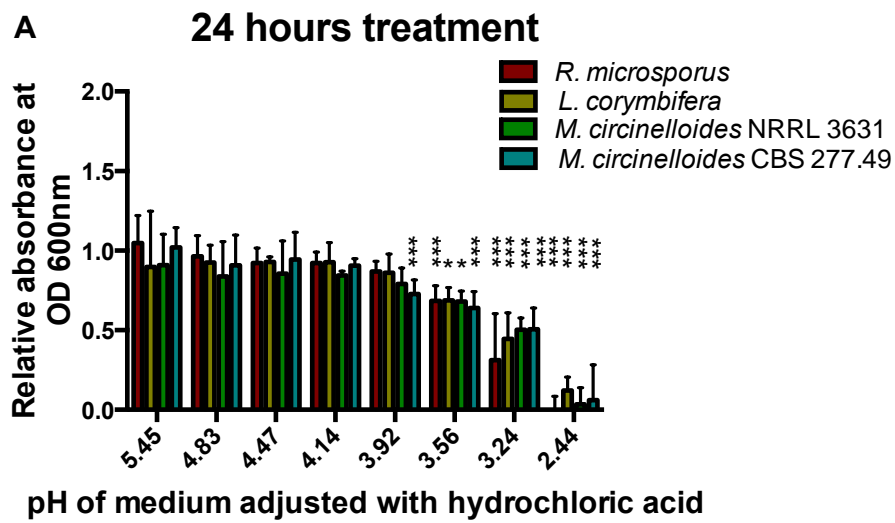
Antifungal activity against *Mucormycetes* is not seen with other acids.

To investigate whether this effect is driven solely by environmental pH, we performed similar experiments using an alternative organic acid (lactic acid) and an inorganic acid (hydrochloric acid). The pH of sabouraud media seen at different concentrations of acetic acid was matched with either hydrochloric or lactic acids (Table 2.1) and monitored for spore germination. Neither hydrochloric nor lactic (Figure 2.5) acids significantly inhibited spore germination other than at the very lowest pHs. Thus, the antifungal effect of acetic acid is not simply a reflection of lowered environmental pH.

Acetic acid in sabouraud media		Acetic acid in RPMI media	
Percentage	pH	Percentage	pH
5	2.52	5	2.32
2.5	3.27	2.5	3.19
1.25	3.63	1.25	3.67
0.63	3.92	0.63	4.03
0.31	4.18	0.31	4.46
0.16	4.43	0.16	5.24
0.08	4.68	0.08	6.32
Sabouraud (control)	5.73	RPMI (control)	7.45

Table 2.1 pH of acetic acid in media.

pH of acetic acid in either Sabouraud (left) or RPMI (right) media.



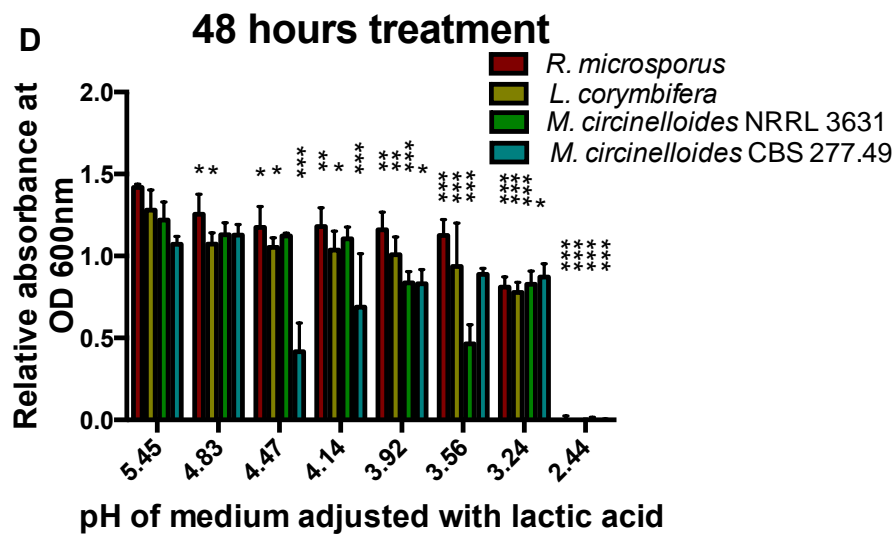


Figure 2.5 Lack of inhibition of spore germination by other acids.

Other acids are less effective at inhibiting spore germination. Neither hydrochloric (A-B) nor lactic (C-D) acids are able to inhibit spore germination as effectively as acetic acid. Spores were grown in sabouraud media with addition of either hydrochloric (A-B) or lactic (C-D) acids and growth assessed by OD 600_{nm} measurements. Graphs show OD measurements after 24 and 48 hours. Error bars represent standard deviation (n=3, three experimental replicates at each time) and statistical analysis was conducted using two-way ANOVA with Dunnett's multiple comparison test. *p < 0.05, **p < 0.01, ***p < 0.001.

Growth inhibition by acetic acid involves both pH and acetate dependent effects.

To test a potential pH-independent effect of acetic acid, we neutralised different concentrations of acetic acid by adding NaHCO_3 to return the pH to that of the control, but retaining the presence of free acetate. These pH-neutralized media continued to inhibit spore germination (Figure 2.6) less effectively than non-neutralized acetic acid, but more effectively than other acids. Thus, the suppression of fungal germination by acetic acid involves pH-dependent and pH-independent, but acetate-dependent, mechanisms.

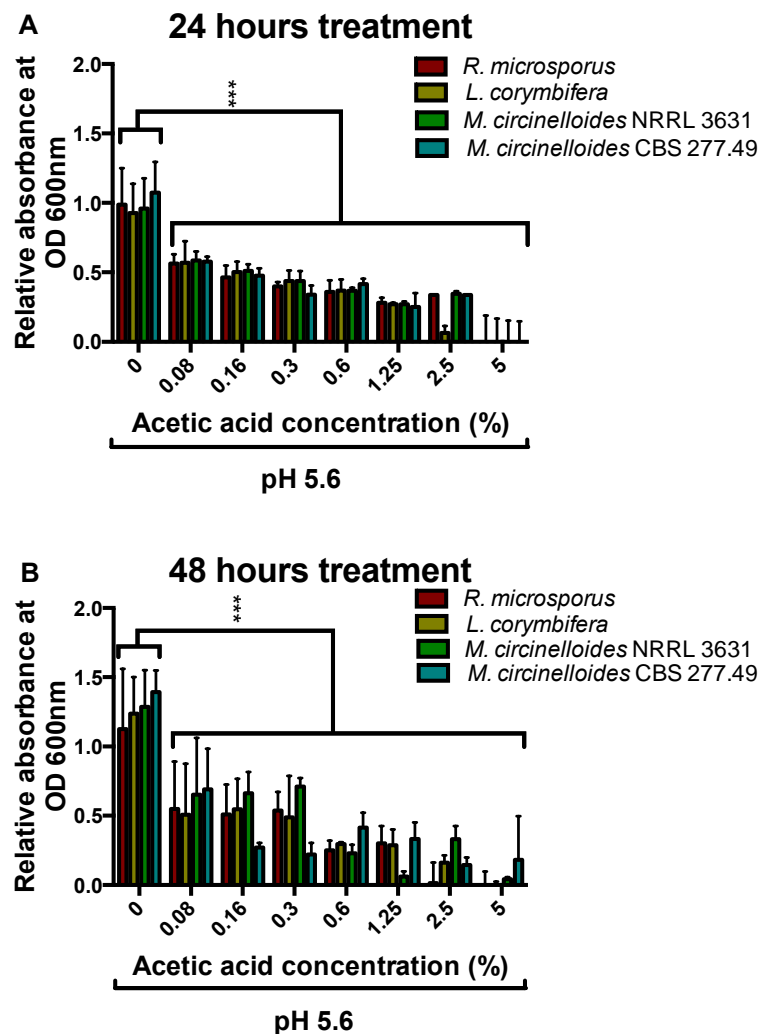


Figure 2.6 Neutralised acetic acid solutions still inhibit fungal germination.

Neutralized acetic acid inhibits fungal spore germination with moderate efficacy. Spores were grown in sabouraud media with different concentrations of acetic acid adjusted to pH 5.6 with sodium bicarbonate and growth assessed by OD 600_{nm} measurements. Graphs show OD measurements after 24 (A) and 48 (B) hours. Error bars represent standard deviation (n=3, three experimental replicates at each time) and statistical analysis was conducted using two-way ANOVA with Dunnett's multiple comparison test. *p < 0.05, **p < 0.01, ***p < 0.001.

Acetic acid inhibition of spore germination is not solely due to reduced intracellular pH.

To investigate the mechanism by which acetic acid inhibits spore germination, we measured (experiment performed by Dr. Joao N. Correia) the intracellular pH of fungal spores during exposure to acetic, hydrochloric, and lactic acid. Cells were incubated with the pH-responsive BCECF-AM dye and monitored following acid exposure. At identical extracellular pHs, acetic acid lowered intracellular pH more strongly than hydrochloric or lactic acids, likely due to stronger dissociation of the ions within the fungal cytoplasm (Figure 2.7). However, even at identical intracellular pHs, acetic acid is far more effective at inhibiting fungal germination, suggesting that this inhibition is not solely due to the raised intracellular hydrogen ion concentration (e.g., compare growth inhibition by 0.3 % acetic acid in Figure 2.1 with growth inhibition by hydrochloric acid at pH 3.56 in Figure 2.5, both of which drive an intracellular spore pH of 5.9, as shown by the results in Figure 2.7).

Data produced by Dr. Joao N. Correia (University of Birmingham, May lab)

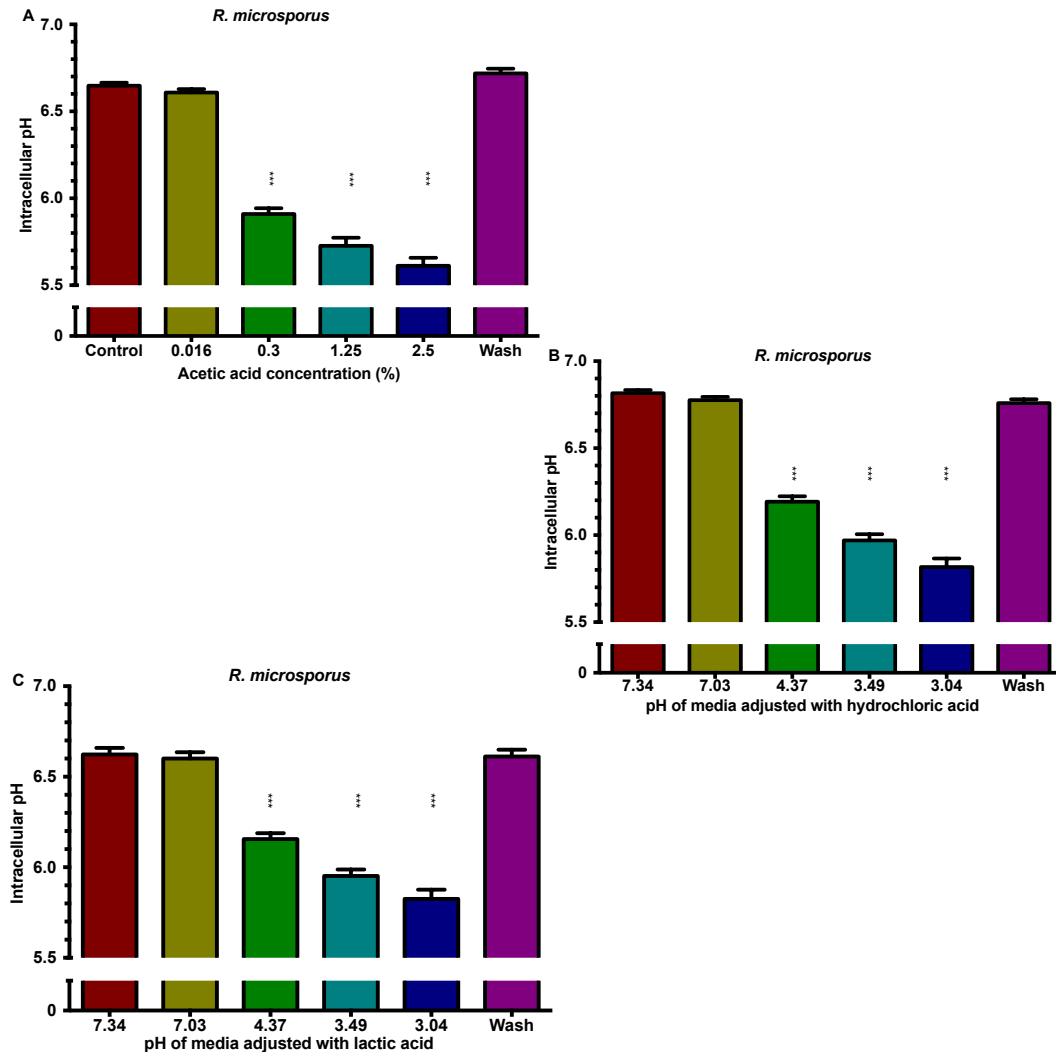


Figure 2.7 Intracellular pH measurement in *R. microsporus*.

Intracellular pH measurements following treatment with different acids and, following experimentation, upon return to neutral pH via a wash with buffered medium. Spores were grown in RPMI medium supplemented with different acids. The intracellular pH was measured using the calibrated ratiometric analysis of the BCECF-AM dye described in Materials and Methods. Error bars represent standard errors of the means ($n = 3$, with three experimental replicates at each time point); statistical analysis was conducted using two-way ANOVA with Dunnett posttest. ***, $P < 0.001$.

Acetic acid shows activity against actively growing *Mucormycetes*.

In a clinical setting, *Mucormycetes* spores may have germinated before treatment can be initiated. Thus, to investigate whether acetic acid is active on germinated spores filamentously growing fungal cells were monitored by time-lapse microscopy and then exposed to different concentrations of acetic acid (Figure 2.8). As with non-germinated spores, growing fungi were strongly inhibited by acetic acid concentrations as low as 0.3 % (Figure 2.8). Thus, acetic acid is effective even on spores that have already germinated.

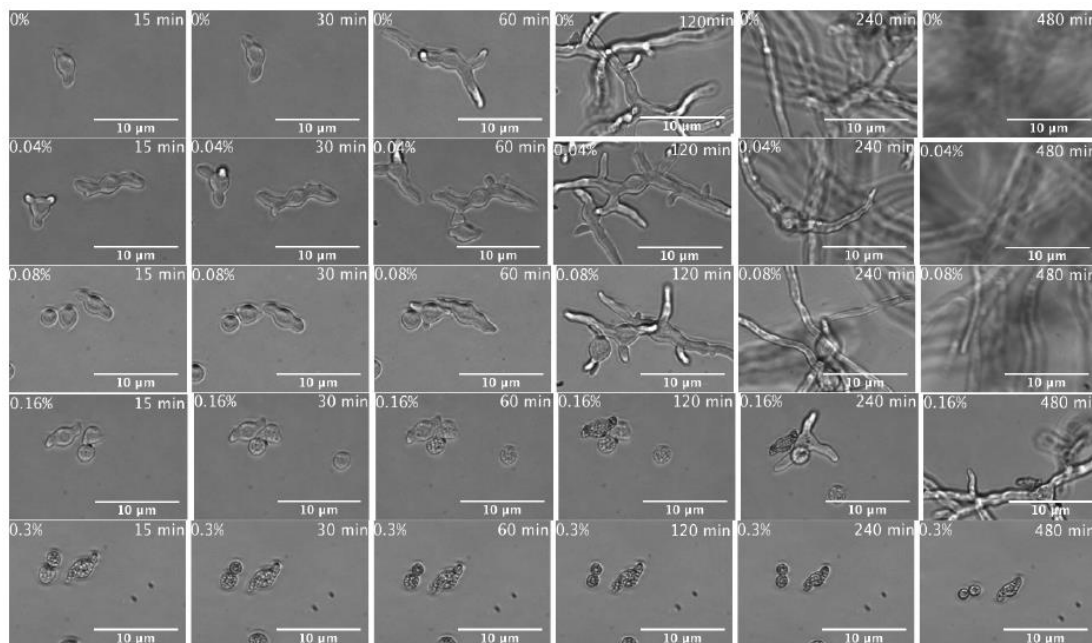


Figure 2.8 Acetic acid antifungal activity on germinating spores.

Time-lapse imaging shows prolonged inhibition of further fungal growth in germinated spores at acetic acid concentrations of 0.3 % or above. Acetic acid percentage (%) is presented in the corner of each image.

Acetic acid shows strong antifungal activity against *Scedosporium*, *Lomentospora* and *Fusarium* species.

Scedosporium, *Lomentospora* and *Fusarium* fungal spores are the infectious agents for scedosporiosis and fusariosis respectively. To test whether spore germination of *Scedosporium*, *Lomentospora* and *Fusarium* was inhibited by acetic acid, spores of *Scedosporium apiospermum* IHEM 14462 and *Lomentospora* (*Scedosporium*) *prolificans* IHEM5608; *Fusarium oxysporum* IHEM 25499 and *Fusarium solani* IHEM 6092, were inoculated into sabouraud broth (to mimic nutrient-rich conditions) with varying acetic acid concentrations. Time-lapse imaging was performed on fungal spores to verify fungal inhibition of germination by acetic acid (Figure 2.9-2.12).

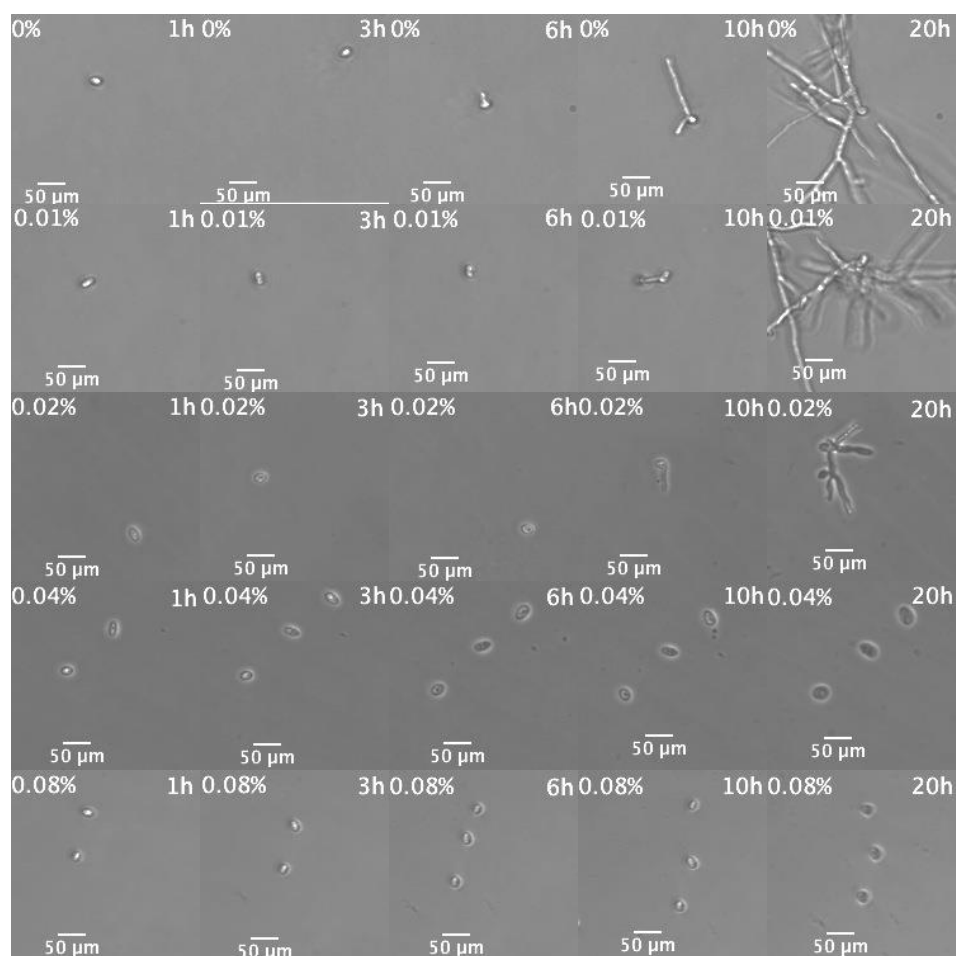


Figure 2.9 Visualization of acetic acid activity against *S. apiospermum* IHEM 14462.

Time-lapse imaging shows acetic acid concentrations of 0.4 % strongly inhibit germination. Acetic acid percentage (%) is presented in the corner of each image.

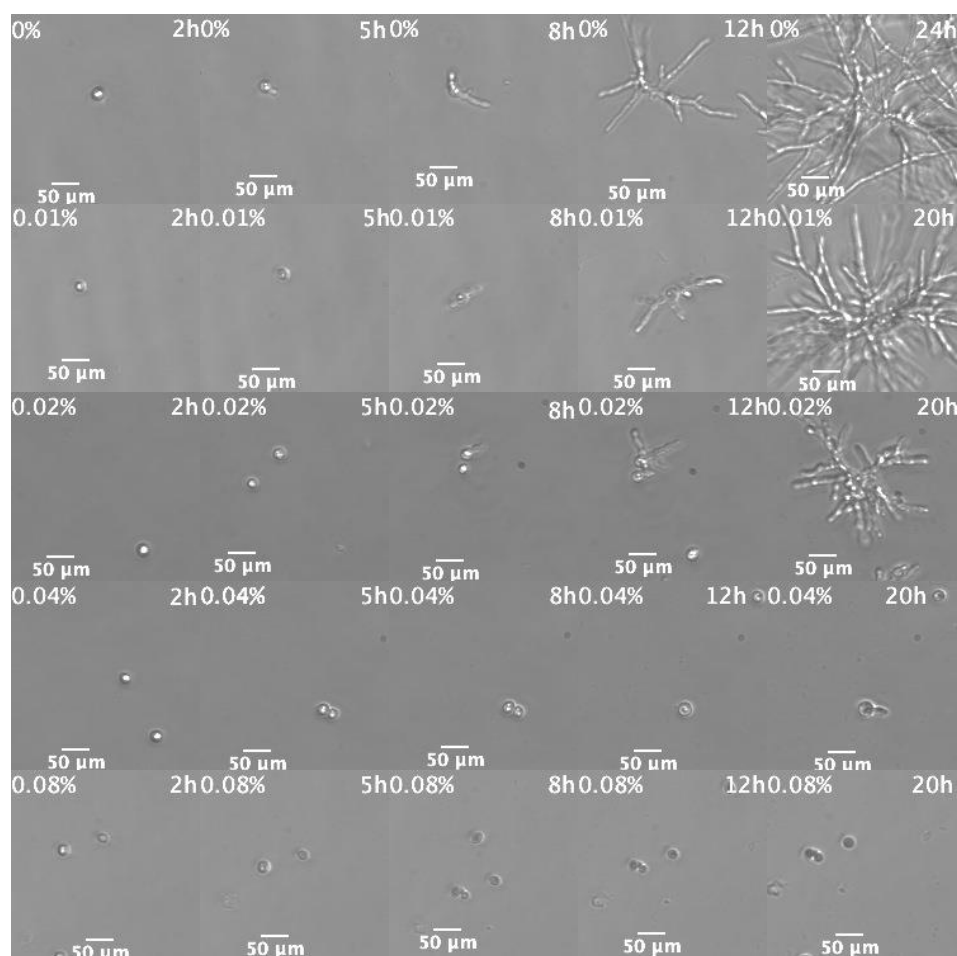


Figure 2.10 Visualization of acetic acid activity against *L. prolificans* IHEM 5608.

Time-lapse imaging shows acetic acid concentrations of 0.4 % strongly inhibit germination. Acetic acid percentage (%) is presented in the corner of each image.

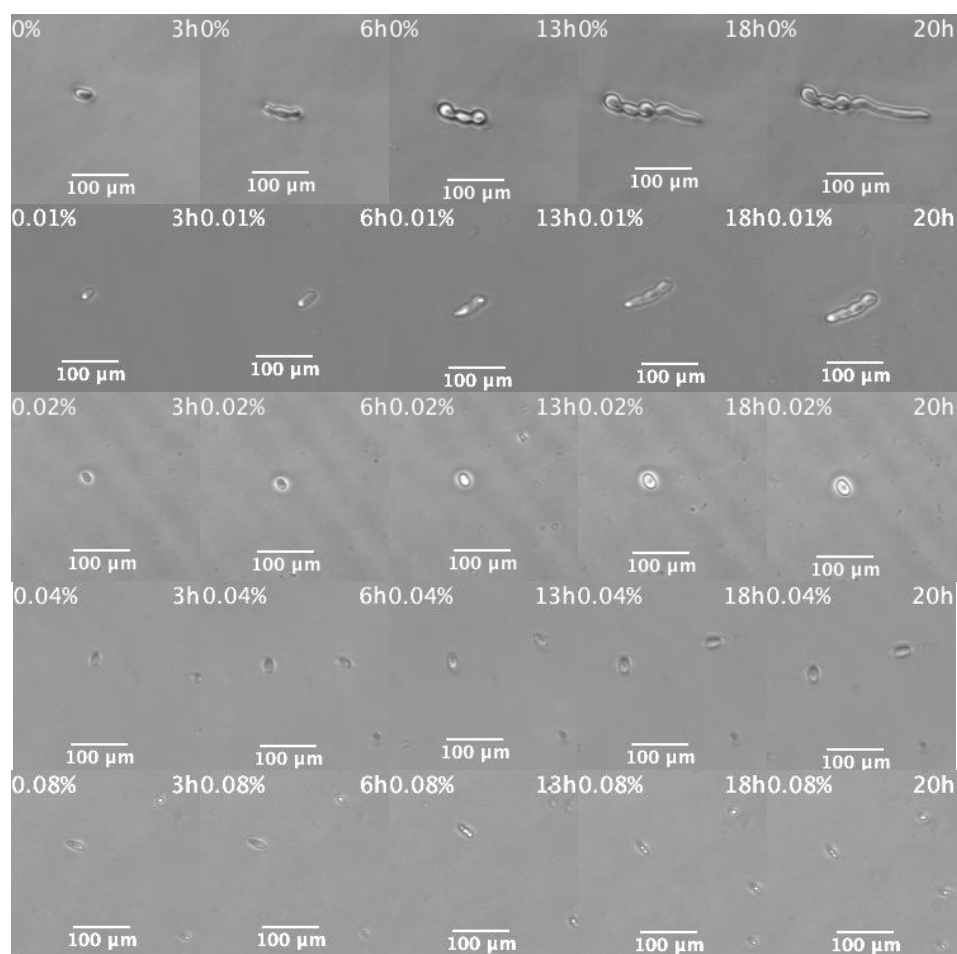


Figure 2.11 Visualization of acetic acid activity against *F. oxysporum*.

Time-lapse imaging shows acetic acid concentrations of 0.01 % strongly inhibit germination. Acetic acid percentage (%) is presented in the corner of each image.

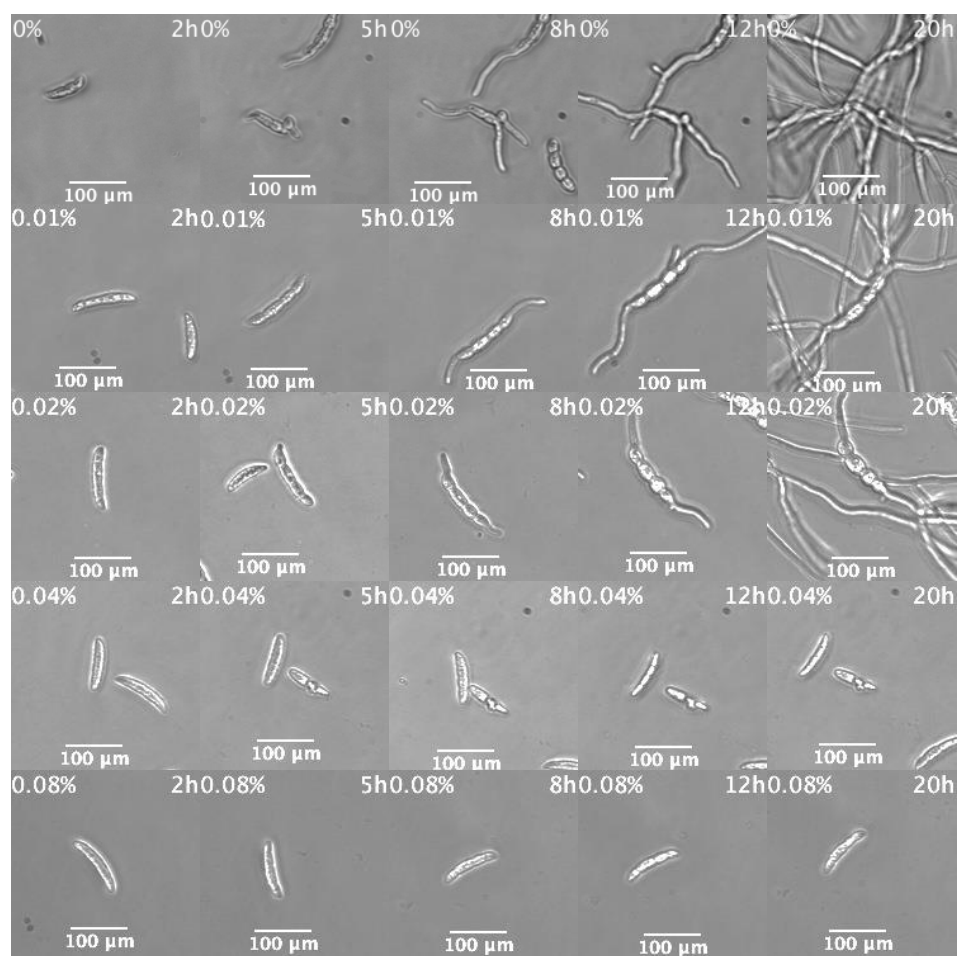


Figure 2.12 Visualization of acetic acid activity against *F. solani*.

Time-lapse imaging shows acetic acid concentrations of 0.04% strongly inhibit germination. Acetic acid percentage (%) is presented in the corner of each image.

Acetic acid shows strong antifungal activity against *Candida* and *Cryptococcus* species.

First, the concentration of acetic acid that kills *Cryptococcus* and *Candida* cells were tested. For *Cryptococcus* spp., fungal cells were incubated in different acetic acid concentrations and then plated on agar plates for CFU counting. The results show that concentration of acetic acid as low as 0.4 % killed *Cryptococcus* cells (Fig. 2.15). For *Candida* spp. cells were inoculated in different acetic acid concentrations and observed under time-lapse microscopy. The results show that for both isolates of *C. albicans* SN152 and DAY286 tested, acetic acid concentrations as low as 0.3 % are sufficient to inhibit fungal growth (Fig. 2.13-2.14).

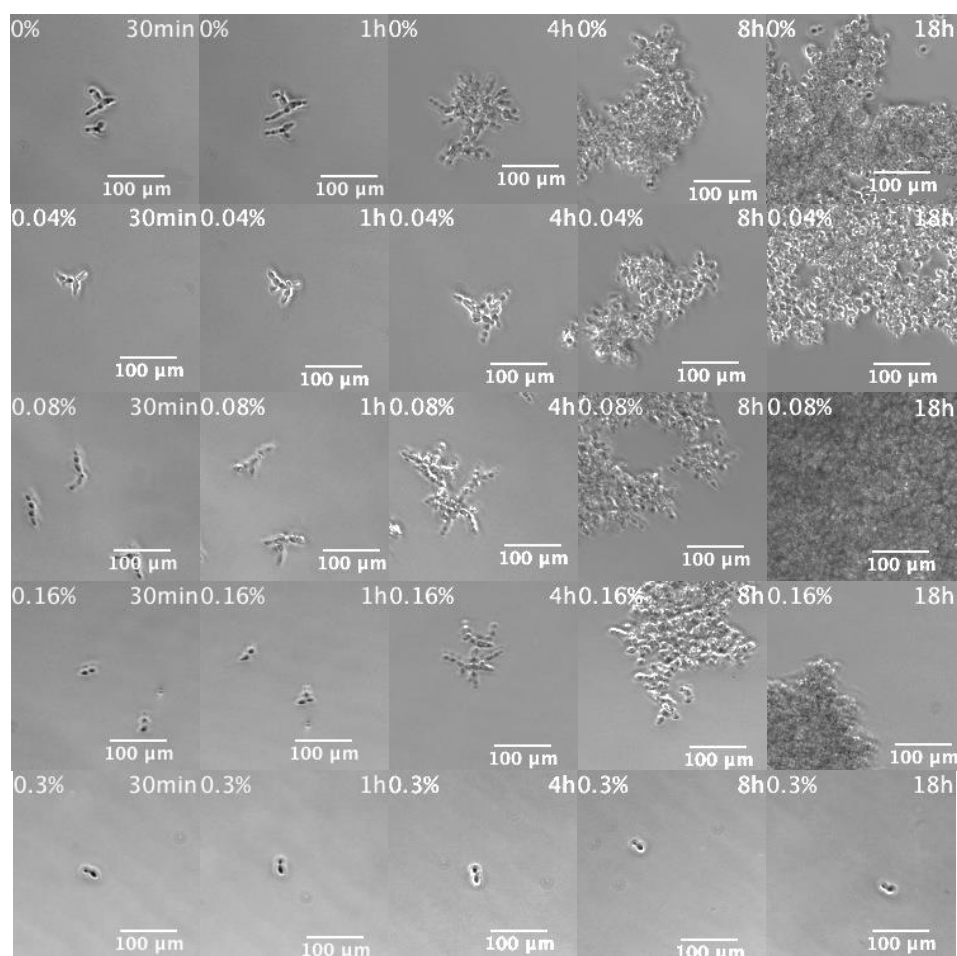


Figure 2.13 Visualization of acetic acid activity against *C. albicans* SN152.

Time-lapse imaging shows acetic acid concentrations of 0.3 % strongly inhibit growth.

Acetic acid percentage (%) is presented in the corner of each image.

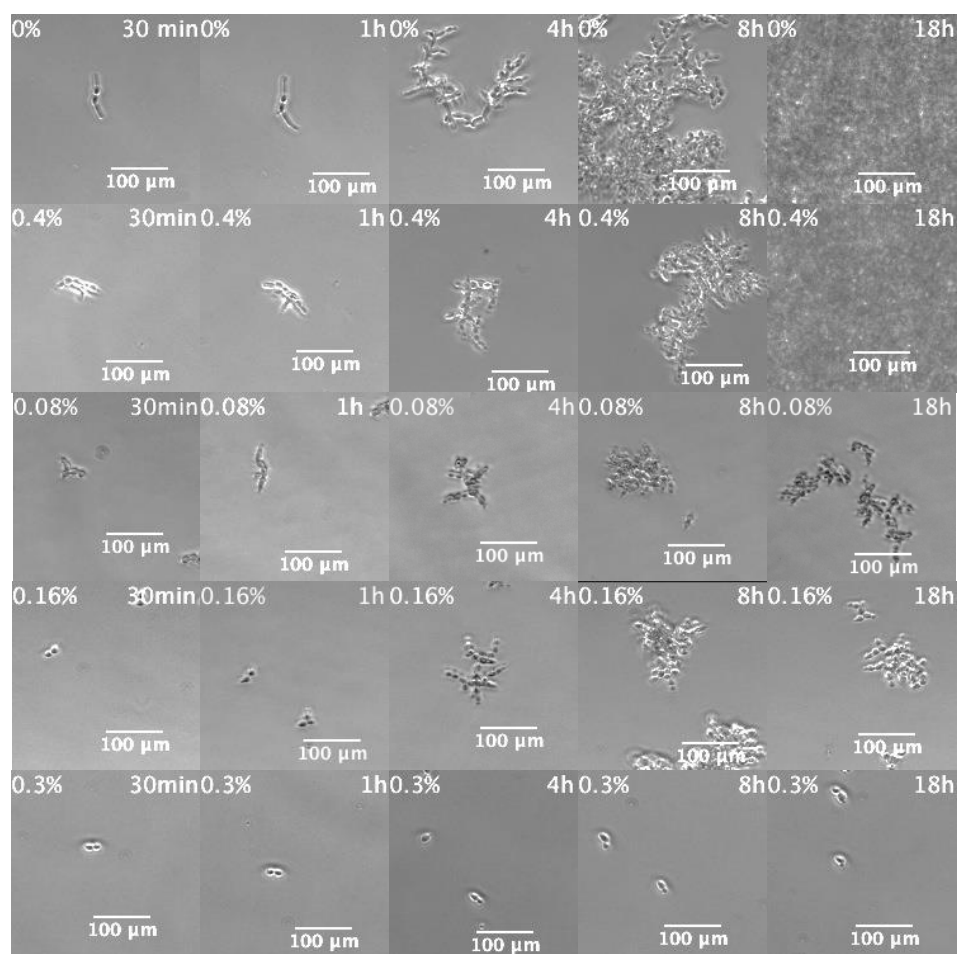


Figure 2.14 Visualization of acetic acid activity against *C. albicans* DAY286.

Time-lapse imaging shows acetic acid concentrations of 0.3 % strongly inhibit growth.

Acetic acid percentage (%) is presented in the corner of each image.

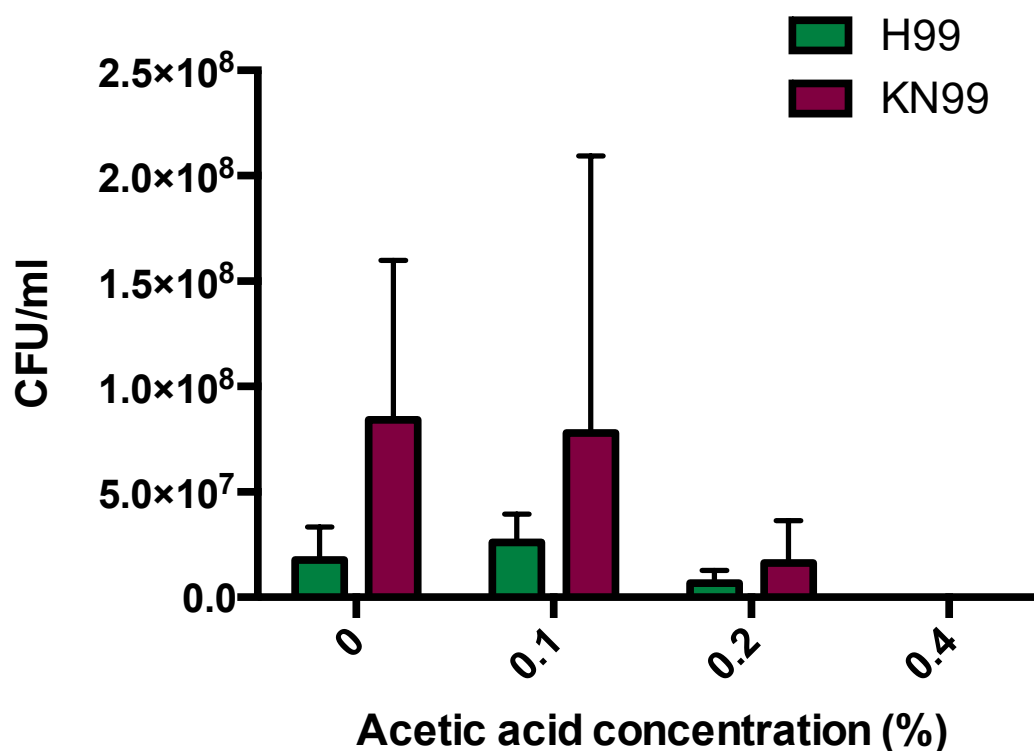


Figure 2.15 *C. neoformans* H99 and KN99 strains survival after acetic acid treatment.

CFU counting shows that 0.4 % of acetic acid is fungicidal to both strains of *C. neoformans* tested. Yeast cells were grown in YPD media for 48 hours supplemented with different concentrations of acetic acid and plated onto YPD agar plates. Number of colony forming units was counted 24 hours later. Error bars represent standard deviation (n=3, three experimental replicates at each time) and statistical analysis was conducted using two-way ANOVA with Dunnett's multiple comparison test. *p < 0.05, **p < 0.01, ***p < 0.001.

2.4 Discussion

In this chapter I have described the fungistatic and fungicidal properties of acetic acid against several *Mucormycete*, *Scedosporium*, *Lomentospora*, *Fusarium*, *Candida* and *Cryptococcus* species.

Fungal infections are a common complication of traumatic injuries sustained in both military and civilian environments like agricultural, motor vehicle, and natural disasters or blunt crush injuries (Warkentien et al., 2012). All trauma-associated fungal infections caused by fungi tested in this project are a cause of life-threatening infection, with high mortality rates (Church et al., 2006; Evriviades et al., 2010; Warkentien et al., 2012). Whilst susceptible patient groups (e.g. those with impaired immunity and traumatic injuries) are increasing, diagnosis is often delayed and antifungal therapy typically ineffective and expensive (Pfaller and Diekema, 2004; O'Donnell et al., 2008; Lackner et al., 2012). Moreover, fungal tissue infections are very difficult to treat as many of these species show intrinsic resistance to antifungals and drug accessibility to wounded tissue is poor (Wuthisuthimethawee et al., 2015). Hence, there is a need for prompt, prophylactic treatment for high-risk patients (e.g. severely injured patients). Thus, acetic acid represents a novel, potentially powerful strategy for dealing with such infections.

Here we have tested and demonstrated that very low concentrations of acetic acid inhibit spore germination and yeast growth. Levine and Fellers (1940) early studies on food spoilage microorganisms such as *S. aureus*, *B. cereus*, *S. cerevisiae* or *A. niger*, suggested that toxicity of acetic acid is not only due to the dissociated hydrogen ion alone but also seems to be functioning in conjunction with the undissociated acid. Our results support these observations, showing that acetic acid

is more fungistatic and fungicidal than other acids tested (hydrochloric and lactic, inorganic and organic acids respectively) even if matched for extracellular and intracellular pH. Here, we have investigated by measuring intracellular pH of fungal spores treated with different acids, and by neutralising acetic acid pH have shown that the antifungal effect of acetic acid is not simply a reflection of lowered environmental pH. Therefore, we propose that the antifungal activity of acetic acid is a function of both undissociated acid and/or free acetate, together with the reduced pH driven by the dissociated hydrogen ions. As undissociated acid molecules diffuse through the cell membrane, they dissociate further within the cytoplasm. Thus, the fungus must expend energy both to pump out excess protons and to deal with free acetate (salt) within the cytoplasm.

Clinically, there are considerable benefits in using acetic acid; it is a cheap compound that is non-hazardous and temperature stable. It shows great potential to be used as a topical antifungal treatment in those challenging clinical situations where application to wounded tissue is difficult, for instance, in dressings for military personnel.

As it works at very low concentrations there is a very low risk of patient discomfort or adverse effect. Currently, 2.5 % of acetic acid is being used to treat bacterial infections in burn wound patients (Halstead et al., 2015). We showed that even low percentage (0.3 %) is very effective against all fungal infections tested in this research. Thus, our data presents acetic acid as a low-cost, effective and rapid treatment for a variety of trauma-associated, skin fungal infection. However, this research has strictly focused on external treatment. Currently there is no acetic acid form available that it could be used intravenously or orally. This holds limitations of the

treatment. Therefore, there is a need to investigate and develop acetic acid compound that could be used as an internal treatment of fungal and bacterial infections in the future.

3. BLUE LIGHT AS A NOVEL ANTIMICROBIAL TREATMENT OF WOUND INFECTIONS

Questions

Is blue light antimicrobial against trauma associated fungal species? Can it be use as a treatment of traumatic injury infected with fungal species?

The majority of this chapter has been published in

Trzaska, W.J. et al. (2017) Species-specific antifungal activity of blue light. *Sci Rep*, 7, 4605.

3.1 Introduction

It is well known that light (in particular blue light) plays an important role as an environmental signal for fungi, such as in growth regulation, sexual or asexual reproduction, or pigment formation (Idnurm and Heitman, 2010). *Neurospora crassa* is a model for investigating light sensing in fungi (Chen, Dunlap and Loros, 2010) and work in this species has identified WC-1, WC-2 and VIVID as the primary blue light photoreceptors (Corrochano, 2007). Blue light is a wavelength of light that is the most effective in fungal photomorphogenesis, and Darwin first reported its response in plants in 1881, proposing that plants could grow differentially in response to external stimuli, such as light or gravity (Purschwitz et al., 2006).

However, blue light can also suppress or inactivate bacteria or fungi (Yin et al., 2013). Different light wavelengths combined with photosensitising dyes have been shown to be very effective against various bacteria, fungi, mycoplasma and viruses (Lembo et al., 2009). Such a combination is called photodynamic therapy (PDT). The principle of PDT is to introduce a photosynthetic agent to the patient that is activated and utilized by light in the presence of oxygen (Sudhakara et al., 2012). However, a major limitation for PDT is the need to introduce exogenous photosensitisers inside the pathogen (Zhang et al., 2014). However, several side effects have been reported due to an inappropriate drug type and dose, light wavelength and toxicity (Sudhakara et al., 2012).

Light has also been used in medicine for treatment of psychiatric conditions, such as dementia, seasonal affective disorder or circadian rhythm sleep disorders; for treatment of arthritic pain and cancer (Shirani and St Louis, 2009; Tafur et al., 2010).

Therefore, blue light has become an interesting tool for many researchers as an

antimicrobial treatment and its effectiveness against pathogens that does not require any exogenous photosensitizers. Zhang et al., (2014) have shown that both *Acinetobacter baumannii* and *Candida albicans* (Zhang et al., 2016) are susceptible to blue light treatment in a mouse burn model. Importantly, the authors also showed that microbes are more susceptible to blue light than keratinocytes, suggesting its advantage to use over other currently used topical treatments such as povidone iodine or hydrogen peroxide that are often toxic and ineffective (Drosou et al., 2003). Other pathogens, such as *Trichophyton rubrum* and *Trichophyton mentagrophytes* (Moorhead et al., 2016); *Helicobacter pylori* (Lembo et al., 2009); *Artemisia vulgaris* (Gold et al., 2009); *Bacillus cereus*, *Clostridium difficile* (Vatansever et al., 2013); *Staphylococcus aureus* and *Pseudomonas aeruginosa* (Dai et al., 2012); and more, have also shown susceptibility to blue light treatment.

The proposed mechanism behind the antimicrobial action of blue light is its ability to activate endogenous porphyrins within cells, which causes their photoexcitation, resulting in production of ROS and cell death (Moorhead et al., 2016). Porphyrin molecules, presented within the cell, absorb the light energy and become excited to the triplet state. Then, the excited photosensitizer interacts with either triplet oxygen, singlet oxygen or cell components to form free radicals and radicals' ions (Ramakrishnan et al., 2016).

Here we tested antifungal activity of blue light against trauma associated fungal species. Some of them such as *Mucormycetes* or *Scedosporium* and *Lomentospora* spp. have not been tested before. We have investigated whether blue light treatment would be a suitable approach to treat fungal infected wounds caused by traumatic injuries. We have shown that 60 min blue light treatment (equivalent to 216 J/cm²) has

species-specific antifungal activity against *Mucormycetes*, *Scedosporium*, *Lomentospora*, *Fusarium* and *Candida* spp. tested. Moreover, we have presented that blue light induce morphological switching from hyphae growth into yeast-like growth in *Mucormycetes*, and their enhanced survival following treatment. Additionally, we have noticed increased temperature produced by the blue light machine during treatment.

Therefore, we propose that the blue light approach is associated with more challenges, as our data suggest that it can have both positive and negative impacts on patient outcome, depending on the infecting species. This requires proper diagnosis prior to treatment.

3.2 Materials and Methods

Unless otherwise stated all reagents were purchased from Sigma Aldrich UK.

Fungal strains.

Eight different strains were tested. *Rhizopus microsporus* 12.6652333 (clinical isolate) and *Mucor circinelloides* NRRL3631 (Li, et al., 2011), *Scedosporium apiospermum* IHEM 14462, *Scedosporium prolificans* IHEM 5608, *Fusarium oxysporum* IHEM 25499, *Fusarium solani* IHEM 6092, all from the Belgian Coordinated Collections of Microorganisms, as well as *Candida albicans* SN152 (Noble and Johnson, 2005) and *Candida albicans* DAY 286 (Nobile and Mitchell, 2009) *Mucormycetes* strains were grown on Sabouraud agar plates (VWR), *Scedosporium* and *Fusarium* spp. were grown on Potato Glucose agar plates for at least 10 days at room temperature before use, and *Candida* spp. were grown on Yeast Extract Peptone agar plates for 2 days at 37° C, then grown over night in YPD broth before use.

Temperature measurement. The temperature of treated fungal suspension was measured within 24 well plates or 25 ml glass conical flasks using a submersible aquarium thermometer (ETI, UK). The temperature on media was recorded throughout treatment.

Treatment with blue light. A LED flood array, composed of 144 LEDs (Henkel-Locite, Hemel Hampstead, UK), was used to treat a 10 x 10 cm area using high-intensity blue light (405nm). All experiments on fungal cells were performed by placing samples within the treatment area for 1 hour, providing an equivalent total dosage of 216 J/cm². Dose J/cm² = Irradiance (W) x exposure time (seconds). To control for temperature effects, experiments were either performed in a cold room (4° C) or at room temperature, as indicated. Spores were washed off the agar plates with

phosphate-buffered saline (PBS) (Fisher Scientific), then spun down 1000 x g/ 3 min/ maximum braking, and resuspended in PBS for cell counting in a haemocytometer. Then, 1×10^4 / ml of fungal spores were inoculated into PBS and placed under the treatment area for 60 minutes. Two control conditions were used; one 24 well multi-well plate covered with aluminum foil inside the blue light machine (exposed to temperature, but not blue light) and another 24 well multi-well plate kept outside the instrument during operation (not exposed to either raised temperatures or blue light). Following treatment, each fungal suspension was transferred onto appropriate agar plates and CFU numbers were counted following growth (typically a few days later). Each experiment was performed in technical triplicate and repeated on at least three occasions. For determining an effect of blue light on fungal morphology assay, fungal spores were inoculated onto agar and treated with blue light for 1 hour in the cold room (including foil and room control plates), then left to grow for a few days.

Time-lapse imaging. Time-lapse imaging was performed on fungal spores immediately after treatment with blue light, using a Nikon Eclipse Ti microscope with an extra-long working distance (ELWD) 0.53 20x Ph1 objective camera, in an environmental chamber at 37° C, with no CO₂ for 18 h. Movies for publication were analysed and prepared in NIS Element software (Nikon). For microscopy experiments, fungal spores were inoculated in Sabouraud broth (*Scedosporium*, *Lomentospora* and *Fusarium* spp.) and YPD broth for *Candida* spp.

Light controls for *R. microsporus*. Light controls for *R. microsporus* and *M. circinelloides* NRRL3631 were performed on fungal spores that were washed of the agar plates, inoculated in Sabouraud broth and covered with aluminum foil or exposed

to light. Plates were incubated in room temperature for 10 hours and samples were taken for imaging.

3.3 Results

Blue light shows antifungal activity against some, but not all, fungal species tested.

For most fungal infections the initiating inocula are fungal spores and disease progression depends on germination and subsequent hyphal invasion of tissue. We therefore exposed spores of six common trauma-associated fungal pathogens (*Rhizopus microsporus*, *Mucor circinelloides*, *Scedosporium apiospermum*, *Lomentospora (Scedosporium) prolificans*, *Fusarium oxysporum*, *Fusarium solani*) to blue light treatment and then transferred onto agar plates for CFU counting over the following days. Blue light was highly effective against *Scedosporium*, *Lomentospora* and *Fusarium* species, but showed no inhibitory effect (but rather enhanced survival relative to controls) on the two species of *Mucormycetes* tested (Figure 3.1).

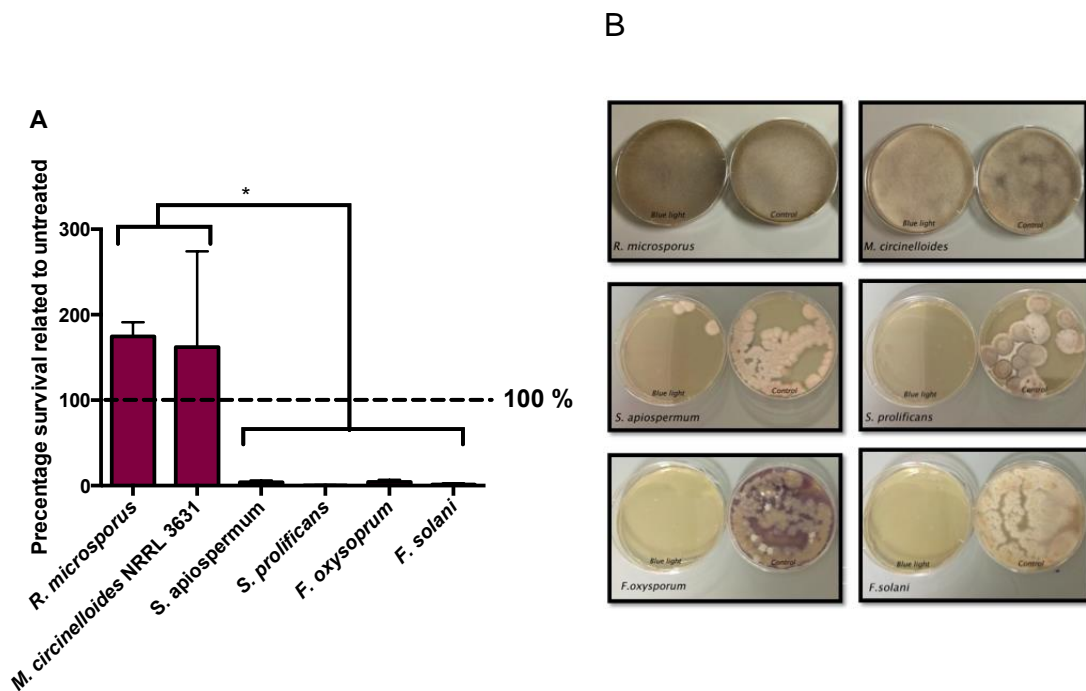


Figure 3.1 60 min (216 J/cm²) blue light treatment of fungal spores.

Spores were inoculated in PBS and treated with blue light and then plated onto appropriate agar plates for enumeration (A). Alternatively, 1000 spores were plated onto agar and then exposed to blue light for 60 minutes before being incubated for growth (B) Error bars represent standard deviation (n=3, with three biological replicates) One-way ANOVA followed by Tukey's multiple comparisons test shows significant difference ($p < 0.05$ for all comparisons) in blue light treatment survival between *R. microsporus* and *M. circinelloides* and other species tested. B) Representative images of fungal growth on agar plates following blue light treatment and 7-10 days of growth at room temperature.

Germination is permanently blocked in most fungal species, but only delayed in *Mucormycetes* and *Candida*.

To visualize blue light effects on fungal pathogens we performed time-lapse imaging on treated fungal spores and additionally included *Candida albicans*, which has previously been shown to be sensitive to blue light killing (Zhang, et al., 2014) (Figure 3.2). Time lapse imaging demonstrated that blue light treatment permanently inhibited germination of *Scedosporium*, *Lomentospora* and *Fusarium* species, but that *Rhizopus microsporus* and *Candida albicans* SN152 eventually recovered full growth capability. Thus, blue light induces a germination/growth arrest that appears permanent in some fungi, but only transient in the *Mucormycetes* and some *Candida* species.

Interestingly, during these studies we also made the chance observation of *R. microsporus* changing its morphology during germination into yeast-like cells that are capable of division by budding (Figure 3.2). Further analysis demonstrated that budding is suppressed by exposure to light (Figure 3.3). Budding-like of this sort has been previously reported for *M. circinelloides* (Omoifo, 2013) but never previously observed in *Rhizopus* species, so this observation raises the possibility that budding-like may be widespread within the *Mucormycetes*.

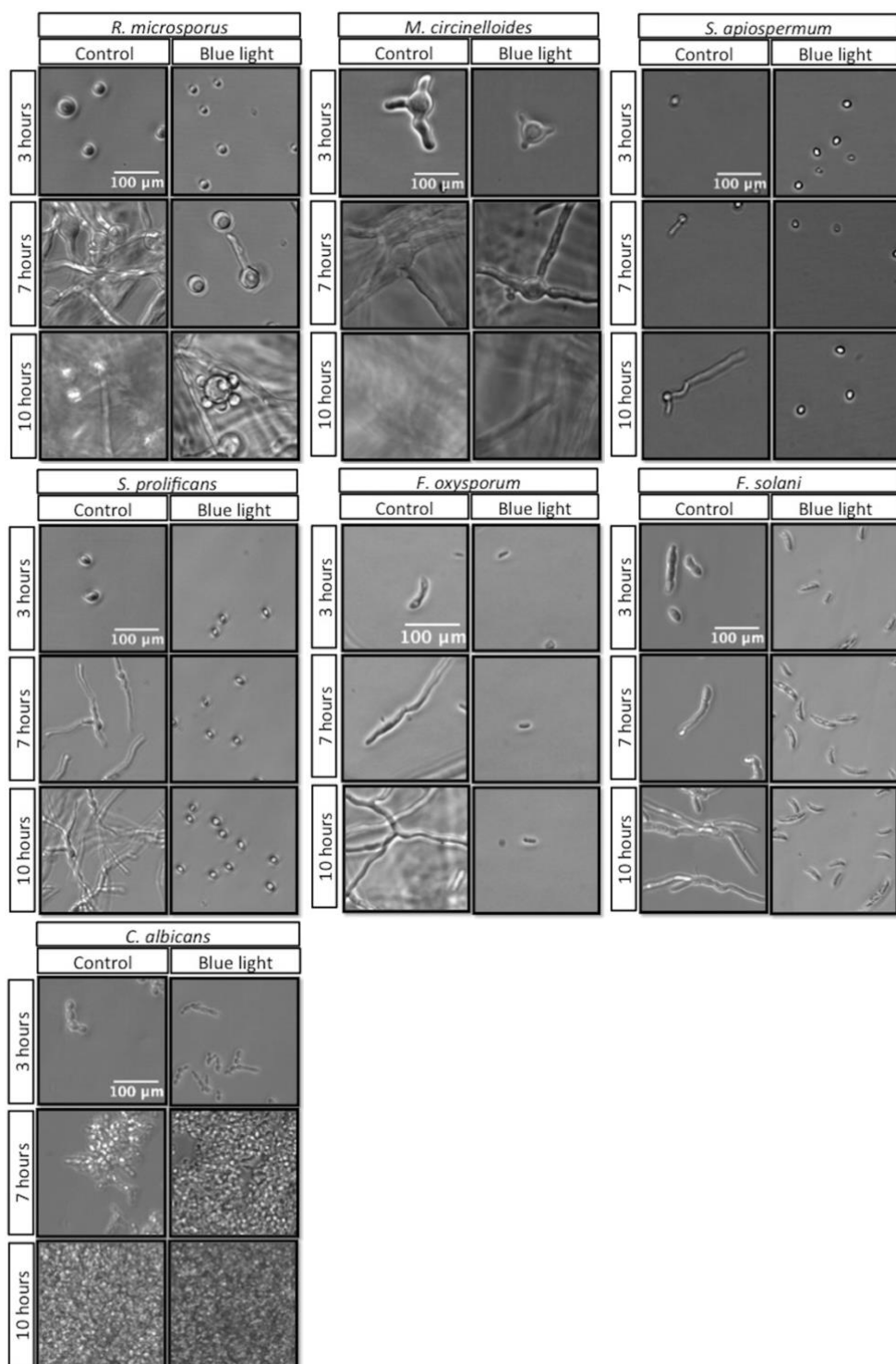


Figure 3.2 Visualization of blue light effect on fungal spores and cells.

Time-lapse imaging shows that blue light 60 min treatment (216 J/cm²) under controlled temperature conditions is effective against *Scedosporium* and *Fusarium* species but not *C. albicans* or *Mucormycetes*.

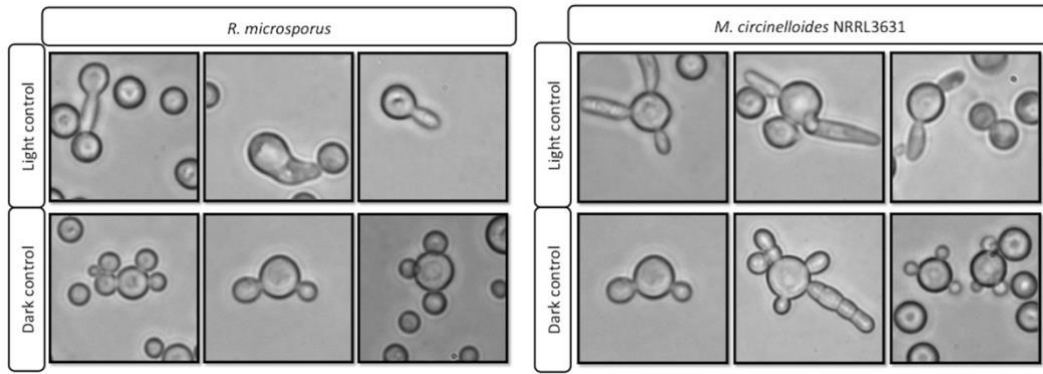
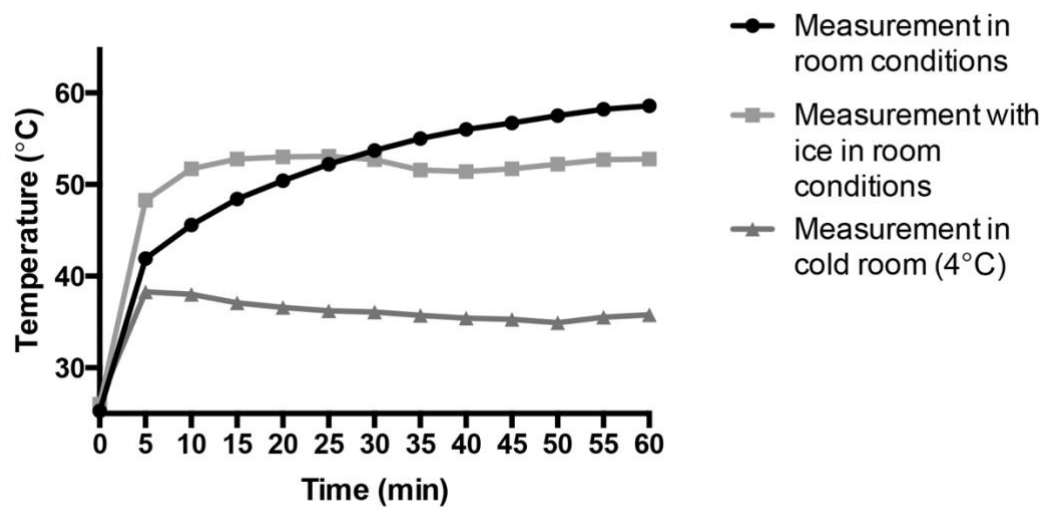


Figure 3.3 *Mucormycetes* switch their morphology to budding-like yeast in the absence of light exposure.

Both *R. microsporus* and *M. circinelloides* NRRL 3631 were washed off the plates and inoculated in sabouraud broth for 10 hours. One sample was exposed to light while the second covered with aluminum foil. Both strains switch their morphology from filamentation to a yeast-like budding when deprived of light during germination-conducive conditions.

Blue light exposure leads to secondary heating, but this is not a major contributor to the growth inhibition effect.

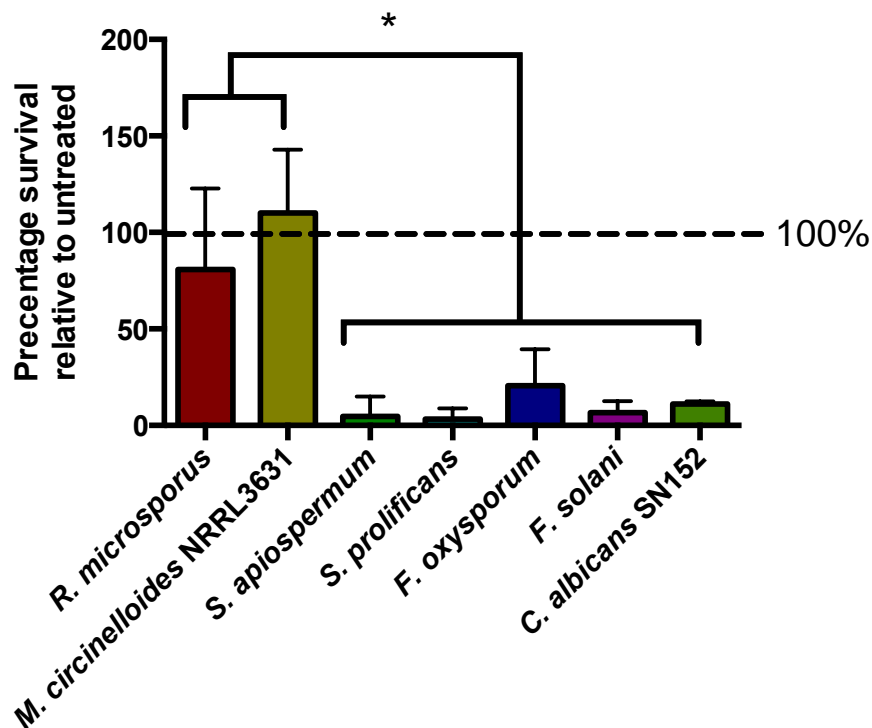
During our investigations we noted that treated samples were significantly warmer than untreated controls. We therefore measured temperature within the medium for samples within the blue light instrument that were either exposed to blue light or wrapped within foil. In both cases, we noted a very rapid increase in temperature during instrument operation (Figure 3.4). Such temperatures are likely to be deleterious to fungal spore survival and we therefore repeated our blue light treatment experiments by housing the instrument within a cold room, which limited the maximum temperature experienced to 37° C (Figure 3.5), a temperature that is fully permissive for growth of these pathogens. When we repeated this assay under these conditions, blue light retained its potent inhibitory effect on the *Fusarium* and *Scedosporium*, *Lomentospora* species and, as before, showed no inhibition of *Mucormycete* survival (Figure 3.5).



(This graph has been produced by Helen Wrigley, 2016).

Figure 3.4 Temperature rises rapidly inside the blue light instrument during operation.

Incubating samples on ice within the instrument is insufficient to reduce this effect, but housing the instrument within a cold room (at 4° C) during treatment maintains sample temperatures at 37° C.



(*C. albicans* data has been produced by Helen Wrigley, 2016).

Figure 3.5 60 min blue light treatment of fungal spores in cold room conditions shows similar growth inhibition effects.

Spores were inoculated in PBS and treated with blue light before plating for growth and CFU enumeration. Error bars represent standard deviation (n=3, with three biological replicates) One-way ANOVA followed by Tukey's multiple comparisons test shows significant difference ($p < 0.05$ for all comparisons) in blue light treatment survival between *R. microsporus* and *M. circinelloides* and other species tested.

Blue light is highly effective against pre-germinated spores.

In clinical settings, fungal infections might have germinated and begun hyphal growth before treatment can be applied. Therefore, we investigated the effect of blue light treatment on pre-germinated spores. Spores were first germinated for 5 hours, and then exposed to 60 minutes of blue light (in cold room conditions to limit temperature exposure to below 37° C) followed by time-lapse microscopy (Figure 3.6). In all cases, blue light treatment effectively stopped further growth of germ tubes, including *Mucormycete* species (which show resistance to blue light as spores, Figure 3.1). Thus, blue light is an effective inhibitory treatment for fungal spores that have already pre-germinated, including for species that are resistant to such treatment as spores.

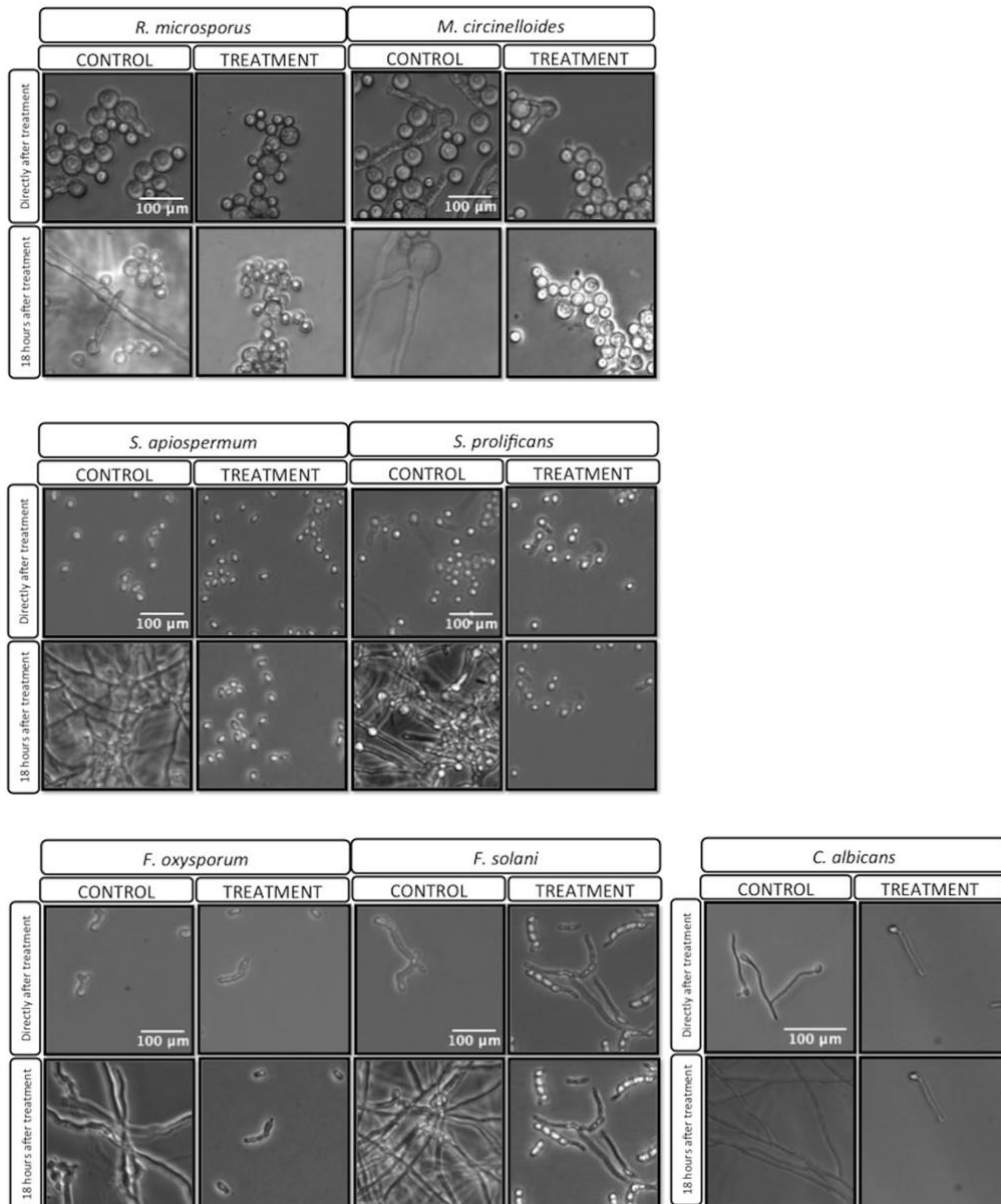


Figure 3.6 60 min blue light treatment show antifungal activity on pre-germinated spores.

Time-lapse imaging shows inhibition of further fungal growth after blue light treatment.

3.4 Discussion

Here we have tested the trauma-associated fungi, including *Mucormyces*, *Scedosporium*, *Lomentospora*, *Fusarium* and *Candida* spp. against blue light therapy.

Fungal tissue infections are very difficult to treat as many of these species show intrinsic resistance to antifungals and drug accessibility to wounded tissue is poor. Thus, blue light may represent a novel approach for dealing with such infections.

We have demonstrated that 60 minutes of blue light treatment, providing an equivalent total dosage of 216 J/cm², shows potent inhibition of fungal growth for spores that have already germinated and produced hyphae in all species tested. In addition, such an approach can also be an effective decontaminant of un-germinated spores for most, but not all, pathogenic species. However, this approach has two notes of caution. Firstly, blue light did not kill the highly antifungal resistant *Mucormycete* species tested, but rather enhanced subsequent germination. In addition, the lack of white light inherent in this device leads species, *R. microsporus* and *M. circinelloides*, to switch morphology, the immunological consequences of which are not known.

Further investigation of interactions between innate immune phagocytes, such as macrophages and neutrophils (first line of defence for fungal pathogens) with the fungal spores that had undergone the morphological switch would help us to gain knowledge about potential virulence and pathogen survival within host. This likely reflects the previously characterized role of blue wavelengths as a regulator of fungal growth (Idnurm & Heitman, 2005). Light that regulates asexual and sexual reproduction as well as pigment formation and direction of growth is a very important aspect of fungal survival within the environment, adaptation and dissemination (Idnurm & Heitman, 2005).

Secondly, we note that the high-intensity blue light system used in our work and by others (Halstead et al., 2016) generates considerable heat. This factor is important to control for when assessing its efficacy against pathogenic microbes on wounded skin. Thus, blue light offers a potentially powerful antimicrobial approach, but its application in situations where infections with *Mucormycetes* and some *Candida* species are likely should be undertaken with caution.

4. SCEDOSPORIUM AND LOMENTOSPORA INTERACTIONS WITH THE HOST INNATE IMMUNE SYSTEM

Research Aim

Understand the basic interaction of *S. apiospermum* and *L. prolificans* with macrophages.

4.1 Introduction

The *Scedosporium* and *Lomentospora* (previously grouped together as *Scedosporium*) genera consist of two medically important species, *Scedosporium apiospermum*/*Pseudallescheria boydii* (sexual state of *S. apiospermum*) and *Lomentospora prolificans* (formerly called *Scedosporium prolificans*) (Cortez et al., 2008). *S. prolificans* was renamed *L. prolificans* due to its phylogenetic distance from other *Scedosporium* spp. (Luo et al., 2017). These omnipresent filamentous fungi are found in sewage, polluted water and soil, are able to grow in aerobic as well as anaerobic conditions (i.e. a facultative anaerobe; an organism able to make ATP in the presence of oxygen, but capable of switching to anaerobic respiration when oxygen is absent), tolerating 5 % or more salt and/or magnesium chloride and surviving in temperatures as high as 42° C (Guarro et al., 2006).

Spores, the infectious agent, can cause infection of different body parts. The most frequent infections are subcutaneous (Tóth et al., 2017; Vijaya. D et al., 2013; Goldman et al., 2016; Bhagavatula et al., 2014), but systemic infections can also occur following organ transplantation, such as kidney (Rathi et al., 2014), lung (Balandin et al., 2016) or heart (Clement et al., 2015) transplantations. Cystic fibrosis (CF) is also

commonly associated with *Scedosporium/Lomentospora* infections (Schwarz et al., 2017; Hirschi et al., 2012) as well as leukemia (Elsayed et al., 1999). However other invasive organ infections without underlying diseases also occur with these species, such as heart (Kelly et al., 2016), lungs (Holmes et al., 2013; Masukane et al., 2017), nasal (Kishimoto et al., 2017; Alvarez et al., 1995; Patel and Orlandi, 2015), contact lens (Arthur et al., 2001) and near-drowning associated infections (He et al., 2015).

The work of several groups has demonstrated species-specific susceptibility to antifungals within the *S. apiospermum/P. boydii* species complex (Cooley et al., 2007; Lackner et al., 2012; Lackner et al., 2014; Wongsuk et al., 2017). Despite their previous taxonomic similarity, there are huge differences such as distinct clinical features depending on the host immune function and species isolated between the *S. apiospermum/P. boydii* species complex and *L. prolificans* (Cooley et al., 2007). In addition to these inter-species differences, there is also considerable variation in antifungal resistance between clinical and environmental strains (Lackner et al., 2012; Lackner et al., 2014). The only antifungal effective against *Scedosporium* spp. is voriconazole (Alastruey-Izquierdo et al., 2007; Wang et al., 2015; Zeng et al., 2004; Gil-Lamaignere et al., 2002). However, *L. prolificans* has been shown to be highly resistant to many antifungals (Meletiadiis, J. et al., 2000; Pellon et al., 2017; Pellon, A. et al., 2018). The intrinsic antifungal resistance of the *Scedosporium/Lomentospora* complex has driven an investigation of alternative treatments such as: *N*-Chlorotaurine, a long-lived oxidant produced by activated monocytes and granulocytes (Lackner et al., 2015); a monoclonal antibody targeting fungal glycosphingolipids in combination with voriconazole (Rollin-Pinheiro et al., 2014); a combination of posaconazole and itraconazole with calcineurin inhibitor tacrolimus (TCR) and/or the Hsp90 inhibitor 17-

demethoxy-17-(2-propenylamino) geldanamycin (17AAG) that induced apoptosis of *L. prolificans* (Shirazi and Kontoyiannis, 2014); or different application therapy such as combination therapy of terbinafine and itraconazole (Meletiadiis et al., 2000). In addition, novel treatments such as echinocandins with granulocyte macrophage colony-stimulating factor (GM-CSF) (Goldman et al., 2016) or treatment of *L. prolificans* by incorporating voriconazole-loaded cement and spacer into hip replacements (Daniele et al., 2017) have been attempted with varying success.

To aid future development of antifungals against the *Scedosporium*/*Pseudallescheria* and *Lomentospora* species complex, there is a need to understand how those fungi interact with the host immune system. The innate immune system is the first line of defense against potentially fatal fungal infections. Patrolling phagocytes, such as macrophages and neutrophils, ingest invading fungi through recognition of surface structures that comprise the fungal cell wall. Once ingested, the harsh environment of the phagosome is thought to kill and eradicate the pathogen. Few research groups have been investigating interaction of *Scedosporium* and *Lomentospora* spp. with innate immune cells and the interaction of these pathogens with the host it is still very understudied.

One of first phagocytic cells to encounter *Scedosporium* are macrophages, which engulf spores to prevent spore germination (Gil-Lamaignere et al., 2003). Gil-Lamaignere et al. (2002) tested antifungal activities of voriconazole (VRC), intraconazole (ITC) and posaconazole (PSC) in mixtures with human polymorphonuclear leukocytes (PMNs). They showed that PMNs, when combined with antifungals, were able to cause more damage to the hyphae of *S. apiospermum* and *S. prolificans*. (Gil-Lamaignere et al., 2002). Xisto et al. (2015), have shown that

peptidorhamnomannan (PRM) present at the cell wall of *S. prolificans*, could be involved in fungal survival in the host; something that appears to be dependent on the O-linked oligosaccharides within the molecule (Xisto et al., 2015). They showed that adding PRM to macrophages led to dose-dependent inhibition of phagocytosis of spores (Xisto et al., 2015). Moreover, killing of fungal spores was unaffected and PRM triggered TNF-alpha release by macrophages.

Some research groups have been investigating the influence of pH, temperature, CO₂ tension (Mello et al., 2016) on germination of *Scedosporium/Lomentospora* or cell wall changes during spore maturation (Ghamrawi et al., 2014) which add a relevant information about biological processes that can be directly related to antifungal resistance and virulence factors.

Pellon et al. (2018) investigated microglial cells (resident immune cells in CNS (Central Nervous System)) interaction with *L. prolificans*. They showed reduced phagocytosis, the increase of oxidative burst, and the production of pro-inflammatory response in comparison to macrophages such as the murine macrophage cell line J774A.1 or murine BMDM (Bone Marrow Derived Macrophages). Moreover, microglial phagocytosis of *L. prolificans* was also dectin-1 and mannose receptor dependent (Pellon et al., 2018). These findings are very interesting as the pattern recognition receptors (PRRs) involved in recognition of *L. prolificans* are very understudied and still remain an important biological question.

Here we investigated how host environment and innate immune cells such as murine macrophages, human monocyte-derived macrophages and iPSC-derived macrophages response to *Scedosporium/Lomentospora* spp. This research will help us to understand how those highly antifungal resistant human pathogens interact and

manipulate the immune system and help us to gain the knowledge of further developing novel drugs to boost the immune system to fight those fungal species.

4.2 Materials and Methods

Unless otherwise stated all reagents were purchased from Sigma Aldrich UK.

Strains, growth conditions and opsonisation. Strains used in experiments: *Scedosporium apiospermum* IHEM 14462, *Scedosporium apiospermum* IHEM 7656, *Scedosporium apiospermum* IHEM 13945 and *Lomentospora prolificans* IHEM 5608, *Lomentospora prolificans* IHEM 19020, *Lomentospora prolificans* IHEM 18755; *Lomentospora prolificans* albino (no melanin) mutant to WT 3.1 strain of *Lomentospora prolificans* Δ Lppks::hph (Al-Laeiby, A. et al., 2016) kindly supplied by Prof. Christopher Thornton, University of Exeter) and *Cryptococcus neoformans* KN99 alpha (Upadhyay, R. et al. 2017)., and *Cryptococcus neoformans* KN99GFP (Garelnabi et al., 2018). All *Scedosporium* and *Lomentospora* strains were grown on Potato Glucose agar plates for at least 10 days before use at room temperature. *Cryptococcus* spp. were grown on YPD agar plates for 2 days at 25° C, then grown overnight in YPD broth before use at 20 rpm at 25° C. For microscopy experiments, the spore pellet (spores harvested from agar plates by PBS, spun down 1000 x g, 3 min, maximum breaking and acceleration, then resuspended in PBS) of *Scedosporium* and *Lomentospora* spp. was resuspended in serum free DMEM and opsonised, or not, with 10% live Human AB Serum in DMEM or RMPI, depending on the tissue culture cells used, for 1 hour, rotating at room temperature. *C. neoformans* KN99 GFP was inoculated in YPD broth overnight at 25° C, rotating. The following day, cell number was counted, diluted to 1×10^7 / ml cell concentration and opsonised with 10 % Human AB Serum for 1 hour, rotating in room temperature.

Macrophage cell line culture. Murine macrophage-like cell line (J774A. 1) was maintained in DMEM media supplemented with 100 U/ml of Penicillin and 100 U/ml of

Streptomycin, 2 mM of L-glutamine and 10% Fetal Bovine Serum (FBS) in a humidified environment at 37° C with 5 % CO₂, and used between 5 and 15 passages from the time of thawing. On the day of infection, macrophage media was replaced with serum free DMEM (DMEM as before but without FBS serum) one hour before infection.

HMDMs isolation and differentiation. Two densities of Percoll were made. 1.079 density [percoll- 19.708 ml, 11.792 ml of distilled water, 3.5 ml of 1.5 M of NaCl]. 1.098 density: [percoll- 24.823 ml, 6.677 ml of distilled water, 3.5 ml of 1.5 M of NaCl]. 6 ml of 1.079 density percoll was layered in the tubes (Thermo-Fisher Scientific), then 6 ml of 1.098 density percoll was layered under 1.079 density percoll. 6 ml of whole blood from healthy volunteers was collected into blood vacutainers containing anticoagulant, then layered over percoll and spun down at 150 x g/8min/no braking, followed by 1200 x g/12 min/no braking. The monocyte/lymphocyte layer and the neutrophil layer was removed and topped up with RBC lysis buffer in 1/3 ratio (1 l distilled water, 8.3 g/l NH₄Cl, 1 g/l KHCO₃, 0.04 g/l Na₂EDTA 2 H₂O and 2.5 g/l BSA (Bovine Serum Albumin, filter sterilized)), gently inverted for 3 minutes and spun down for 6 min/ at 400 x g/ maximum braking. Cells were then washed twice with (ice cold for monocytes) and (room temperature for neutrophils) PBS. Monocytes were then resuspended in RPMI 1640 with L-Glutamine (Gibco) with 5 % human AB serum, heat inactivated (56 degrees for 30 min) supplemented with 100 U/ml of Penicillin-Streptomycin solution for counting. Then cells were seeded at 0.5 x10⁶ cells/well in a 48 well plate (Grainer) and incubated at 37° C, 5 % CO₂ for 1 h to allow cells to attach to the surface. Following that, differentiation media (RPMI 1640 with L-Glutamine (Gibco) with 5 % human AB serum, heat inactivated supplemented with 100 U/ml of Penicillin- Streptomycin solution + M-CSF 20 ng/ml was incubated for 7-15 days in 37°

C, 5 % CO₂ with media change every 3 days. Neutrophils were resuspended in RPMI 1640 with L-Glutamine (Gibco) supplemented with 100 U/ml of Penicillin-Streptomycin solution and infected straight away.

Induced Pluripotent Stem cells (iPSCs). All steps until macrophages were derived from iPSCs, were done by Dr. Leanne Taylor-Smith, University of Birmingham. iPSCs were maintained in plates with Matrigel and MTeSR1 (Stemcell Technologies). On day 0, cells were seeded into an AggreWell 800 plate (Stemcell Technologies) in mTeSR1 (Stemcell Technologies) medium with 10µM ROCKi (AbCam Biochemicals) in EB media to each well. Following seeding, the AggreWell plate was centrifuged at 100x g for 3 minutes for even distribution of cells in microwells. Cells were incubated in a humidified 37° C, 5 % CO₂ incubator. On days 1-3, mesoderm embryoid bodies were fed (media changed) with EB media (mTeSR (Stemcell Technologies), BMP4 at 50 ng/ml (Invitrogen), VEGF at 50 ng/ml (PeproTech), SCF at 20 ng/ml (Miltenyi). EBs were then harvested out of the aggrewells (by taking up the well contents and transferring to 40 µm cell strainer inverted over a 50 ml centrifuge tube. Cells were washed on the strainer several times with PBS, then the sieve was moved into fresh 50 ml tube and EBs washed off with EB media). EBs were then moved into T75 tissue culture flasks (monocyte factory) and returned to incubator. Monocyte factories were fed weekly with 50 % removal and replacement of factory medium (500 ml XVIVO15 (Lonza), 5 ml/ 500 ml GlutaMax (Gibco), 5 ml/ 500 ml P/S (Gibco), 500 ul/ 500 ml 2-Mercaptoethanol (Gibco), M-CSF at 100 ng/ml (Invitrogen), IL-3 at 25 ng/ml (Invitrogen). Monocytes were harvested from the factory flasks (by collection of supernatant). Monocytes were then seeded at 1 x 10⁷/ml density in 48 well plates and

differentiated into macrophages using medium: 50 ml XVIVO15, 0.5 ml/ 50 ml GlutaMax, 0.5 ml/50 ml P/S, M-CSF at 100 ng/ml for 7-14 days.

Infection of macrophages. The murine macrophage cell line (J774A.1) were seeded in to 24 well plates (Greiner) at a concentration of 1×10^5 cells/ ml, 24 hours before the experiment, in cDMEM media. On the day of experimentation, for all macrophages media was replaced with serum free DMEM (J774s) or Serum free RPMI (iPSC derived macrophages) one hour before infection. Positive spore colonies of *Scedosporium* and *Lomentospora* spp. were harvested from 2-week-old potato glucose agar cultures using PBS and spun down at 1000x g/ 3min/ maximum braking. The spore pellet was resuspended in serum free RPMI 1640 (or DMEM for J774A.1) with L-Glutamine (Gibco) supplemented with 100 U/ml of Penicillin- Streptomycin solution. *C. neoformans* KN99 GFP mCherry was inoculated in YPD broth over night in 25° C, rotating 20 rpm. Before use, spores were counted and opsonised with 10 % Human AB Serum for 1 hour, rotating at room temperature. *Cryptococcus* cells, *Scedosporium* and *Lomentospora* spores were added to the macrophages in 1:10 and 1:5 infection ratio respectively. *Cryptococcus* cells were added 2 hours before infection with *Scedosporium* and *Lomentospora* and washed with PBS three times.

For phagosome acidification experiments, at the time of infection, 50 nM of LysoTracker Red DND-99 (Thermo Fisher) in serum free DMEM was added per well.

For some of the experiments, *Scedosporium* and *Lomentospora* spp. were either heat killed (30 min, 80° C) or 4 % paraformaldehyde (PFA) (VWR) fixed (20 min in room temperature) and then washed in PBS before opsonisation and infection.

XMD17-109 treatment with 1 μ M for duration of 18 hours as added at the time of infection with *Cryptococcus* ((a specific inhibitor of ERK5, a kinase implicated in the

regulation of vomocytosis (Gilbert et al., 2017)) and co-infection with *C. neoformans* KN99 were also used for some of the experiments.

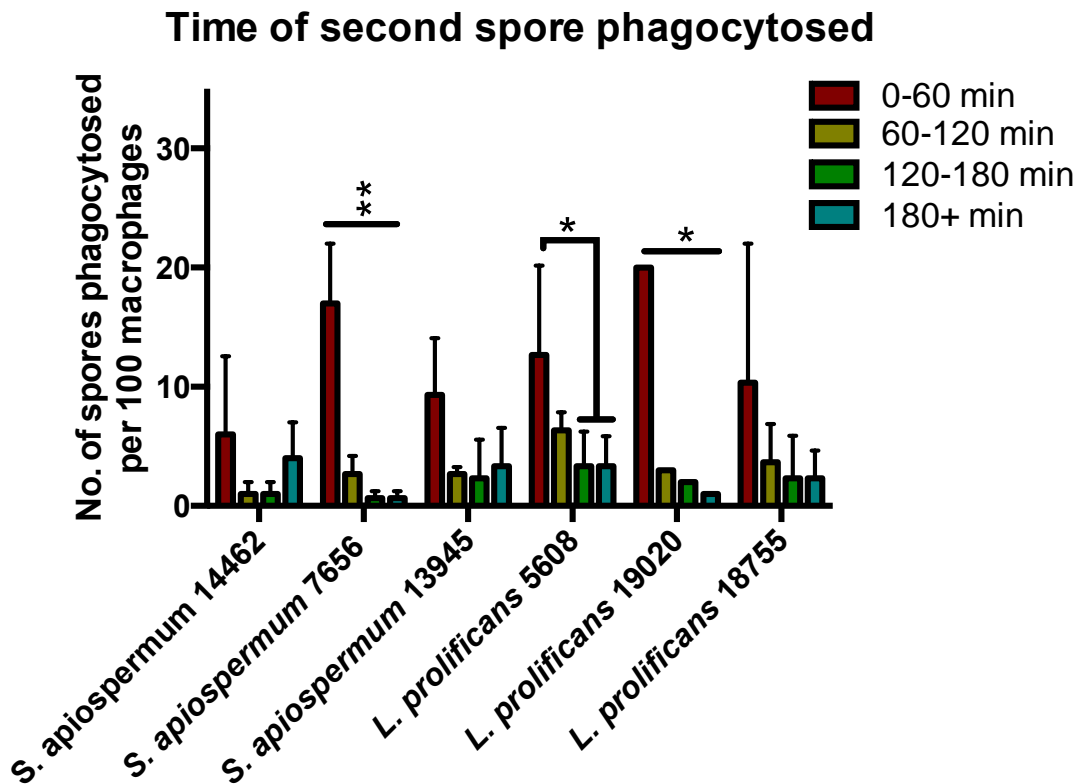
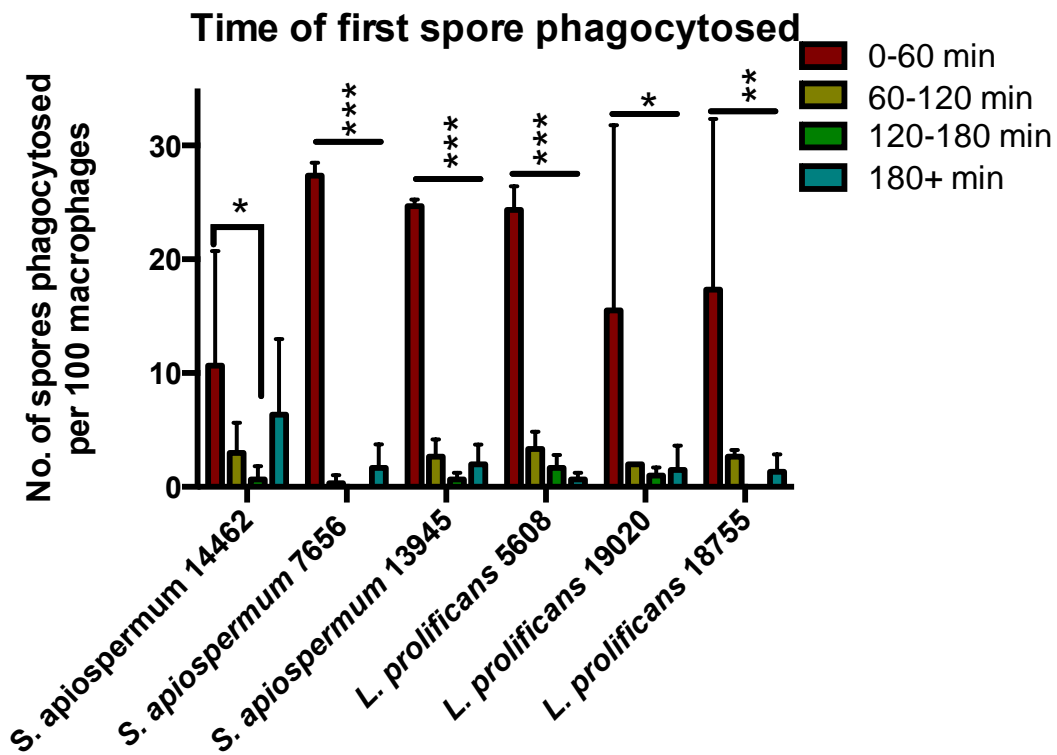
To assess *Scedosporium* and *Lomentospora* spp. survival following phagocytosis, after 2 hours infection cells were washed 3 times with serum free DMEM. Murine macrophages were lysed with distilled water for 20 min in room temperature and samples were plated (after serial dilution) on potato glucose agar plates for CFU count. As a control, spores were also plated without phagocytosis. Colonies were counted once they were large enough to be clearly observed.

Time-lapse imaging was performed on infected macrophages using a Nikon Eclipse Ti microscope with ELWD Ph1 0.52 20x objective for 18 hours with 5 min frame intervals. Camera, QICam (QImaging), environmental chamber with 37° C, 5 % CO₂. Movies were analysed using NIS Elements software.

4.3 Results

Scedosporium and Lomentospora spp. are well phagocytosed by macrophages.

Spores of *Scedosporium* and *Lomentospora* spp. are infectious agents causing infections in both immunocompetent and immunocompromised individuals, resulting in high morbidity and mortality. Innate immune cells are the first line of defense against fungal invasion. We exposed J774A.1 macrophages to non-opsonised spores then counted internalisation. Both species, live and heat-killed, of different isolates of *S. apiospermum* and *L. prolificans* are well phagocytosed, with the majority of uptake occurring within the first 2 hours of infection (Figure 4.1-4.3). Therefore, for further experiments we chose 2 hours infection time to allow for sufficient phagocytosis.



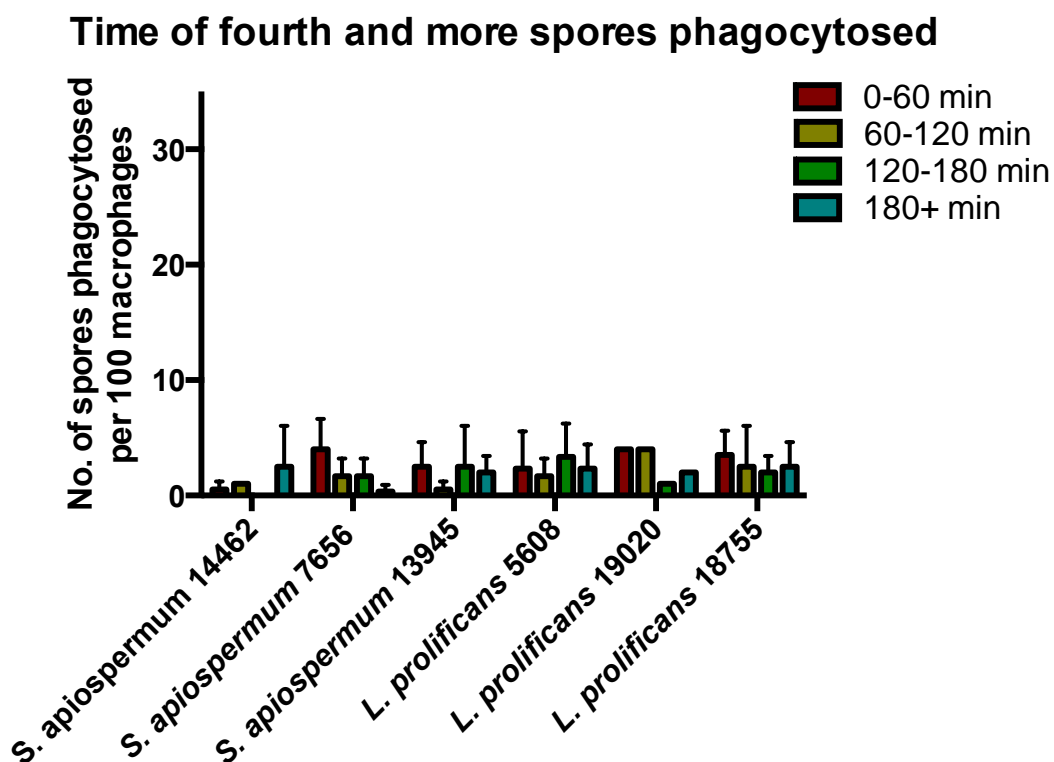
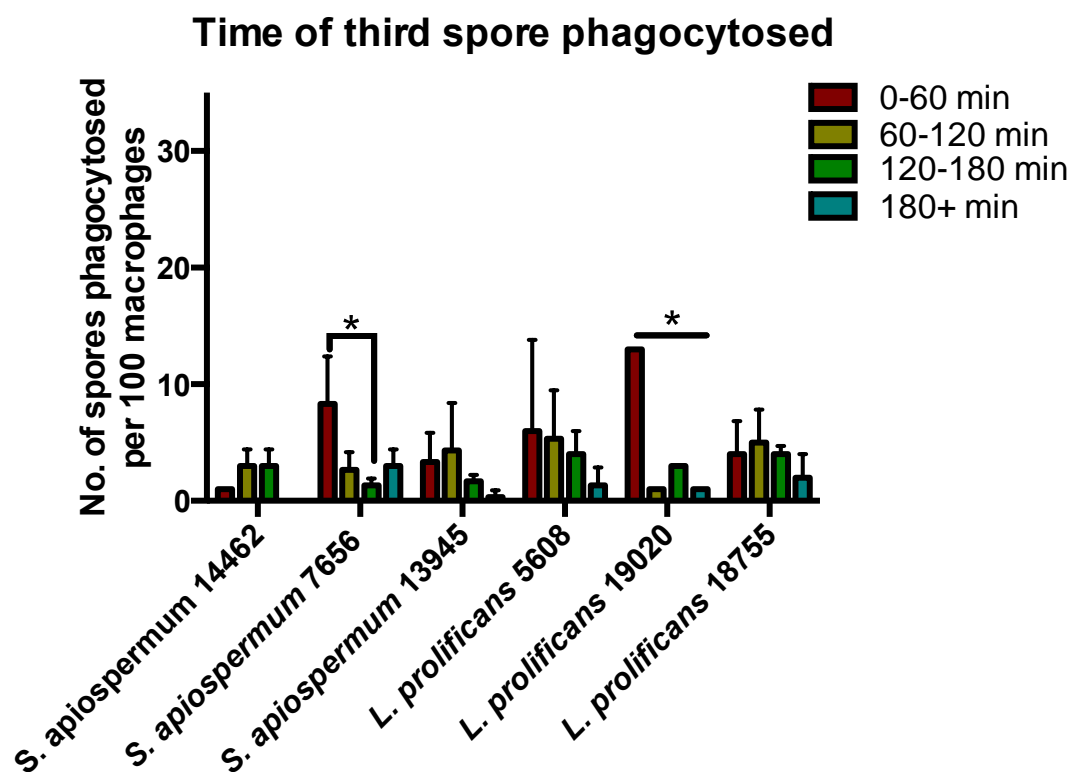
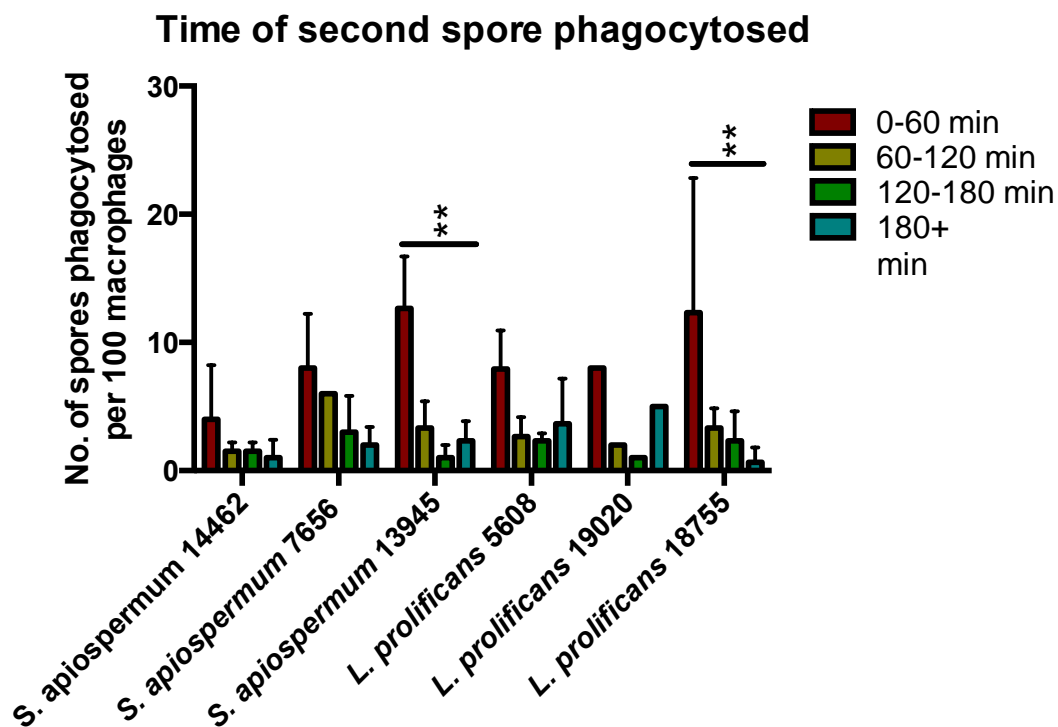
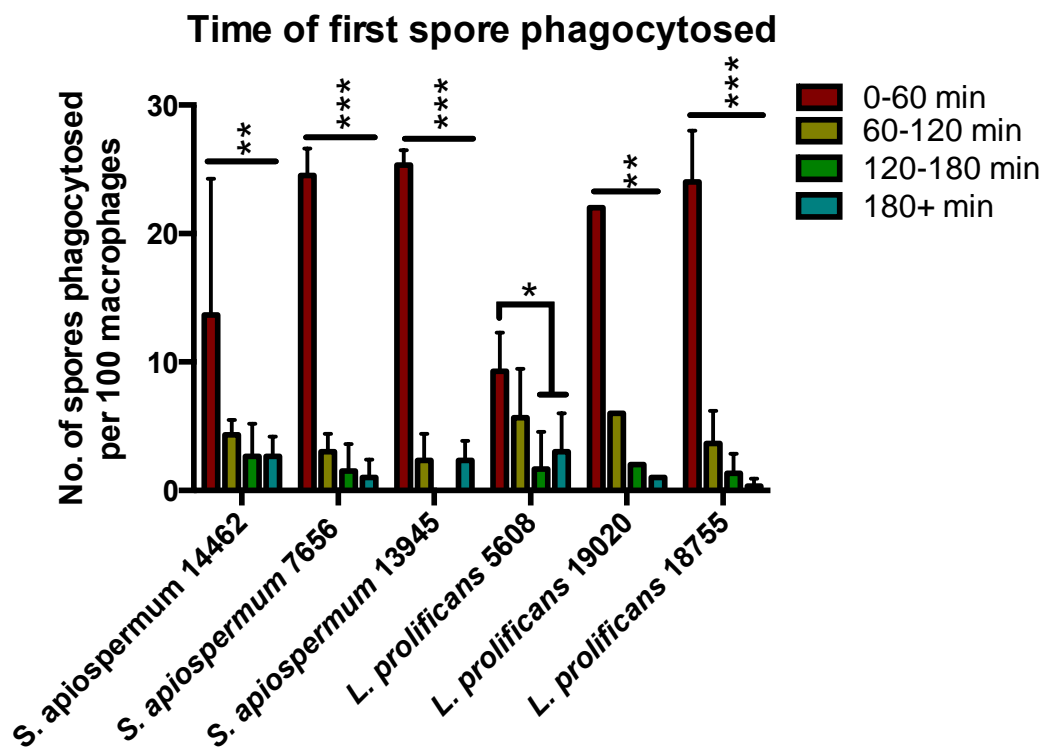


Figure 4.1 Time and number of live spores phagocytosed by murine cell line J774A.1.

The majority of live spores of all isolates of *Scedosporium* and *Lomentospora* spp. were phagocytosed by macrophages within the first 120 minutes. Time-lapse imaging was performed on J774A.1 murine cell line infected with fungal spores using a Nikon Eclipse Ti microscope with LWD 0.52 20x objective for 18 hours. Movies were analysed and phagocytosis scored using NIS Elements software. Error bars represent standard deviation (n=3). Statistical analysis was conducted using two-way ANOVA with Dunnett's multiple comparison test, where all times were compared to first 60 min of phagocytosis. *p < 0.05, **p < 0.01, ***p < 0.001.



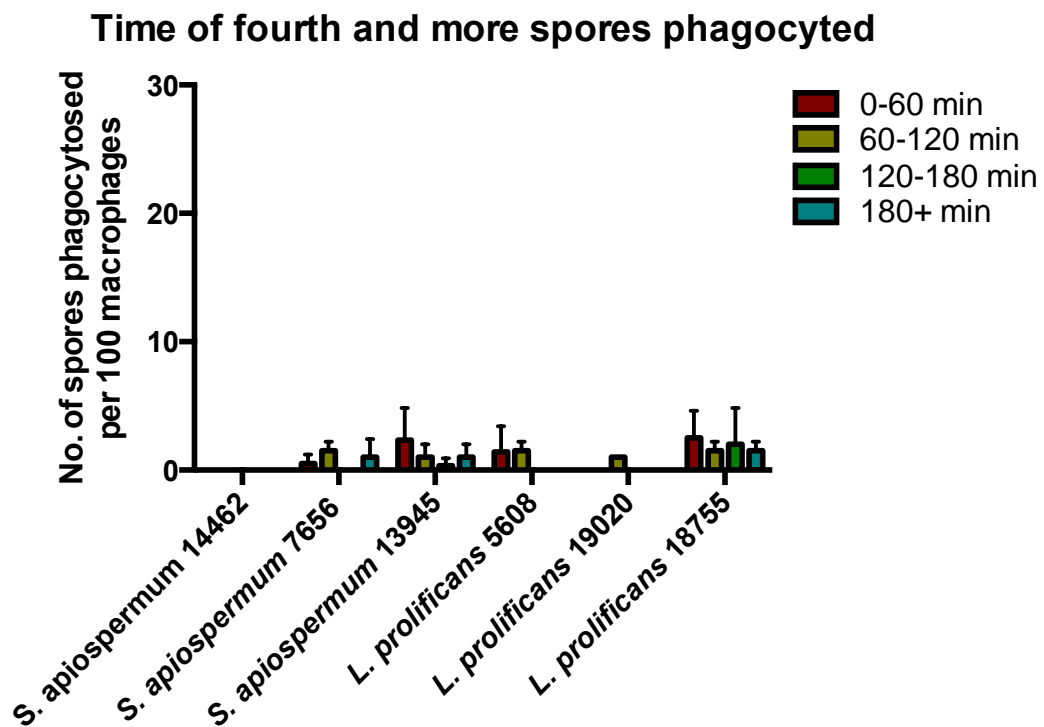
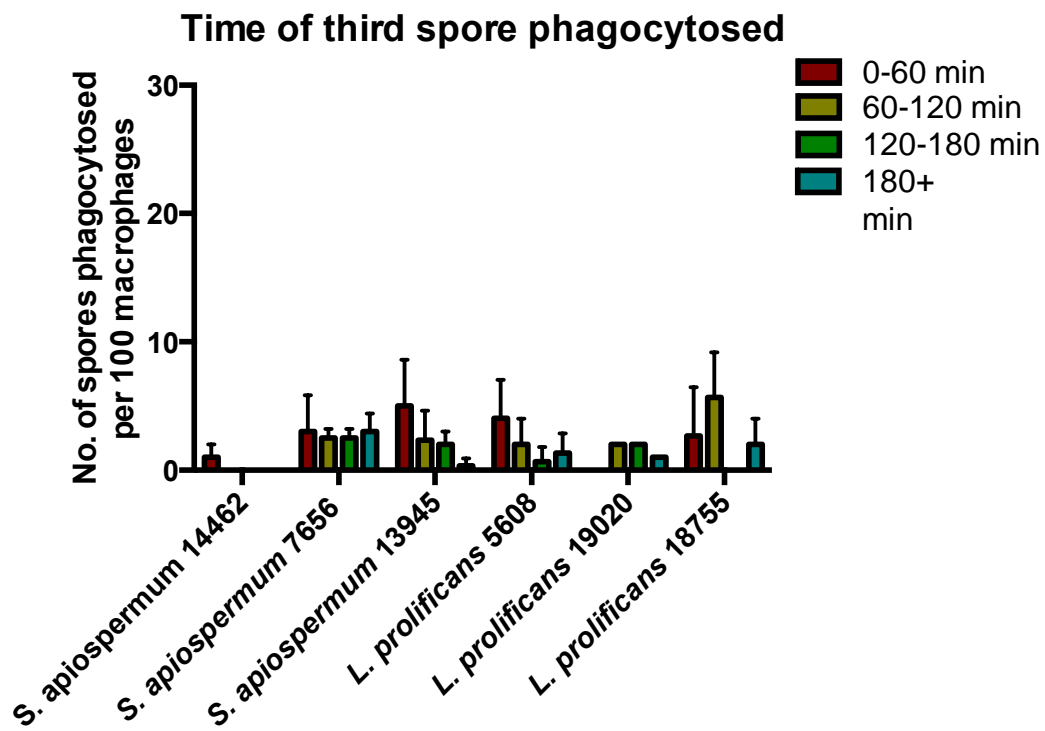


Figure 4.2 Time and number of heat-killed spores phagocytosed by murine cell line J774A.1.

The majority of heat-killed spores of all isolates of *Scedosporium* and *Lomentospora* spp. were phagocytosed by macrophages within first 120 minutes. Time-lapse imaging was performed on J774A.1 murine cell line infected with fungal spores using a Nikon Eclipse Ti microscope with LWD 0.52 20x objective for 18 hours. Spores were heat killed at 80° C for 60 min prior macrophage infection. Movies were analysed and phagocytosis was scored using NIS Elements software. Error bars represent standard deviation (n=3) statistical analysis was conducted using two-way ANOVA with Dunnett's multiple comparison test, where all times were compared to first 60 min of phagocytosis. *p < 0.05, **p < 0.01, ***p < 0.001.

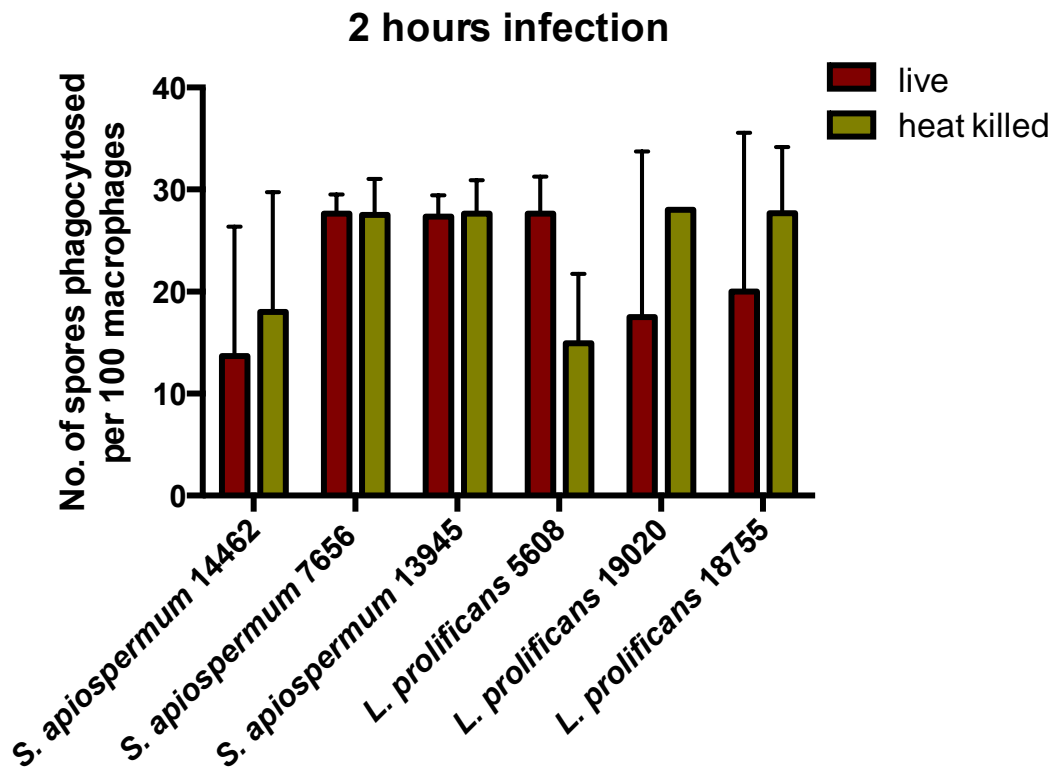


Figure 4.3 Phagocytic rate at 2 hours infection.

Time-lapse imaging was performed on J774A.1 murine cell line infected with fungal spores using a Nikon Eclipse Ti microscope with LWD 0.52 20x objective for 18 hours. Spores were heat killed at 80° C for 60 min prior macrophage infection. Movies were analysed and phagocytosis was scored using NIS Elements software. Error bars represent standard deviation (n=3) statistical analysis was conducted using two-way ANOVA with Dunnett's multiple comparison test, where all times were compared to first 60 min of phagocytosis. *p < 0.05, **p < 0.01, ***p < 0.001.

The murine macrophage cell line is not able to inhibit spore germination.

The innate immune system is the first line of defense against potentially fatal fungal infections. Patrolling phagocytes recognise fungus via their cell wall structure and then ingest and attempt to kill the microbe. However, very little is known of the host defense against *Scedosporium* and *Lomentospora* spp. The primary role of the phagocytic cell is to inhibit spore germination. Therefore, we first investigated whether J774A.1 macrophages are able to inhibit fungal germination following phagocytosis. We exposed J774A.1 macrophages to different isolates of both species and scored germination over an 18 hour time-lapse movie. Germination of *Scedosporium* and *Lomentospora* spp. after phagocytosis by the murine macrophage cell line J774A.1 is very high (up to 80 %), especially for *L. prolificans* isolates. We found a significant difference in spore germination between *S. apiospermum* 14462 and *L. prolificans* 5608 isolates (Figure 4.4), with *L. prolificans* germinating more frequently than *S. apiospermum* within macrophages, therefore we decided to use those two isolates for further experiments.

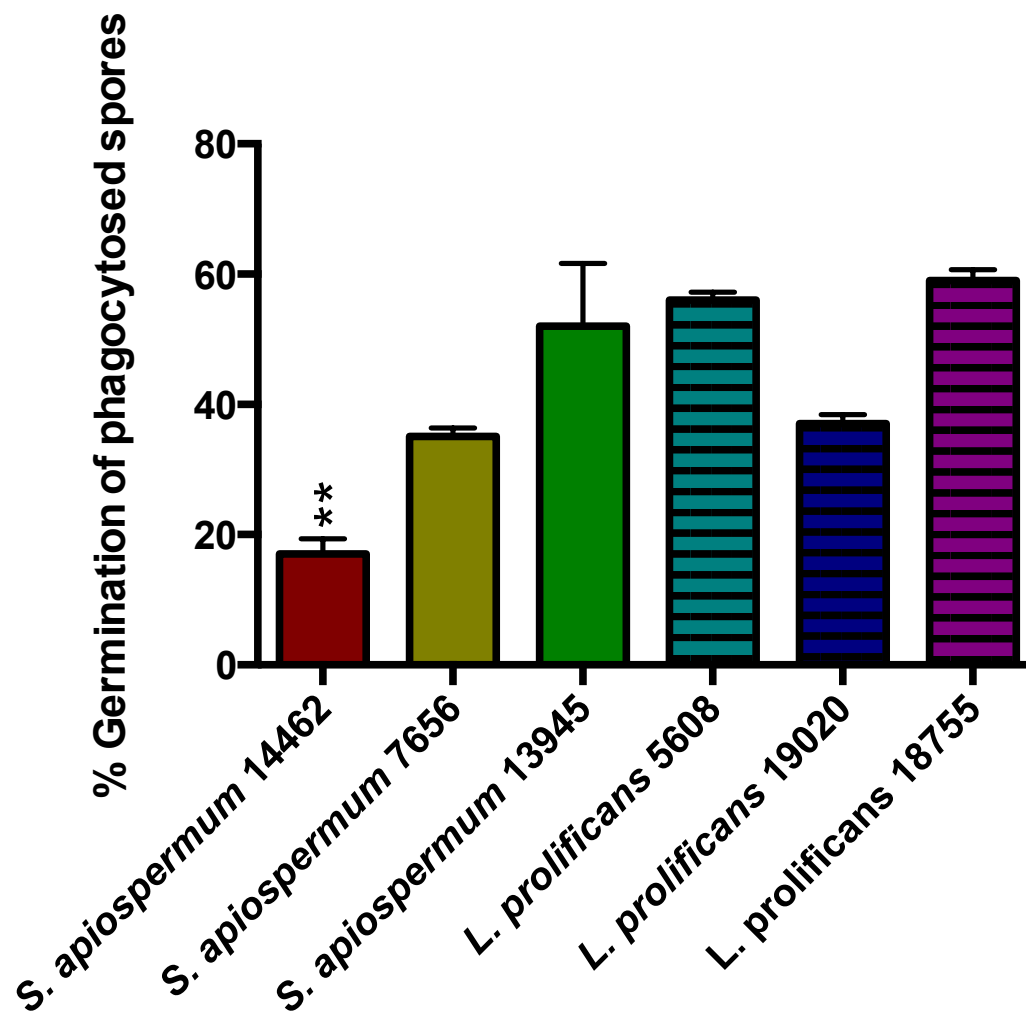


Figure 4.4 Strain and species variation in intracellular germination within J774.A1 macrophages.

Time-lapse imaging was performed and movies were analysed for germination percentage (%) of phagocytosed spores within J774 cells. Error bars represent standard deviation (n=3). Statistical analysis was conducted using two-way ANOVA with Tukey's multiple comparison test. *p < 0.05, **p < 0.01, ***p < 0.001.

The murine macrophage cell line fails to mature the phagosome.

Following successful phagocytosis, macrophages attempt to destroy and eradicate internalised pathogens. To do that, the phagosome undergoes multiple fusion and fission events to generate a toxic environment to destroy pathogens. One of those conditions is increased phagosome acidification, which also serves as a marker of phagosome maturation. After observing that some spores are able to germinate inside J774A.1 cells, we wanted to see if this was because they were able to manipulate phagosome acidification. To test whether phagosomal maturation proceeds normally with these species, we monitored spores that had been internalised by J774A.1 macrophages via time-lapse microscopy. We investigated phagosome acidification by adding 50 nM of LysoTracker Red DND-99 at the time of infection (J774A.1 macrophages exposed to *S. apiospermum* and *L. prolificans* isolates) by either live or heat-killed spores of both strains and scored for acidification over 18 hours of time-lapse microscopy (LysoTracker Red dye selectively accumulates in cellular compartments with low internal pH). There is a noticeable difference (around 60-80 %) between phagosome maturation when macrophages were infected with heat-killed in comparison to live spores (Figure 4.5). These results suggest that both *S. apiospermum* and *L. prolificans* spp. are able to survive within macrophage phagosomes and avoid phagosome acidification enabling them to germinate, penetrate and finally kill host cells. There is no significant difference between live spores and *Scedosporium* and *Lomentospora* spp., however there is significant difference of phagosome acidification between heat killed spores of *L. prolificans* and other strains.

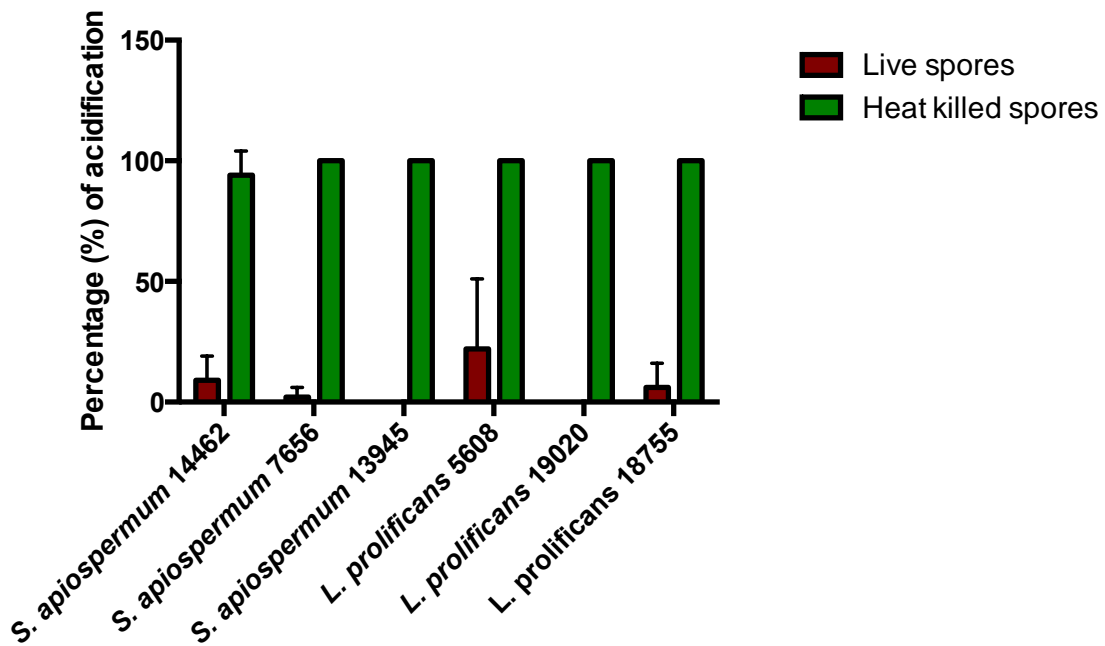


Figure 4.5 Phagosome acidification of murine macrophage cell line J774A.1 followed by *Scedosporium* and *Lomentospora* spp. phagocytosis.

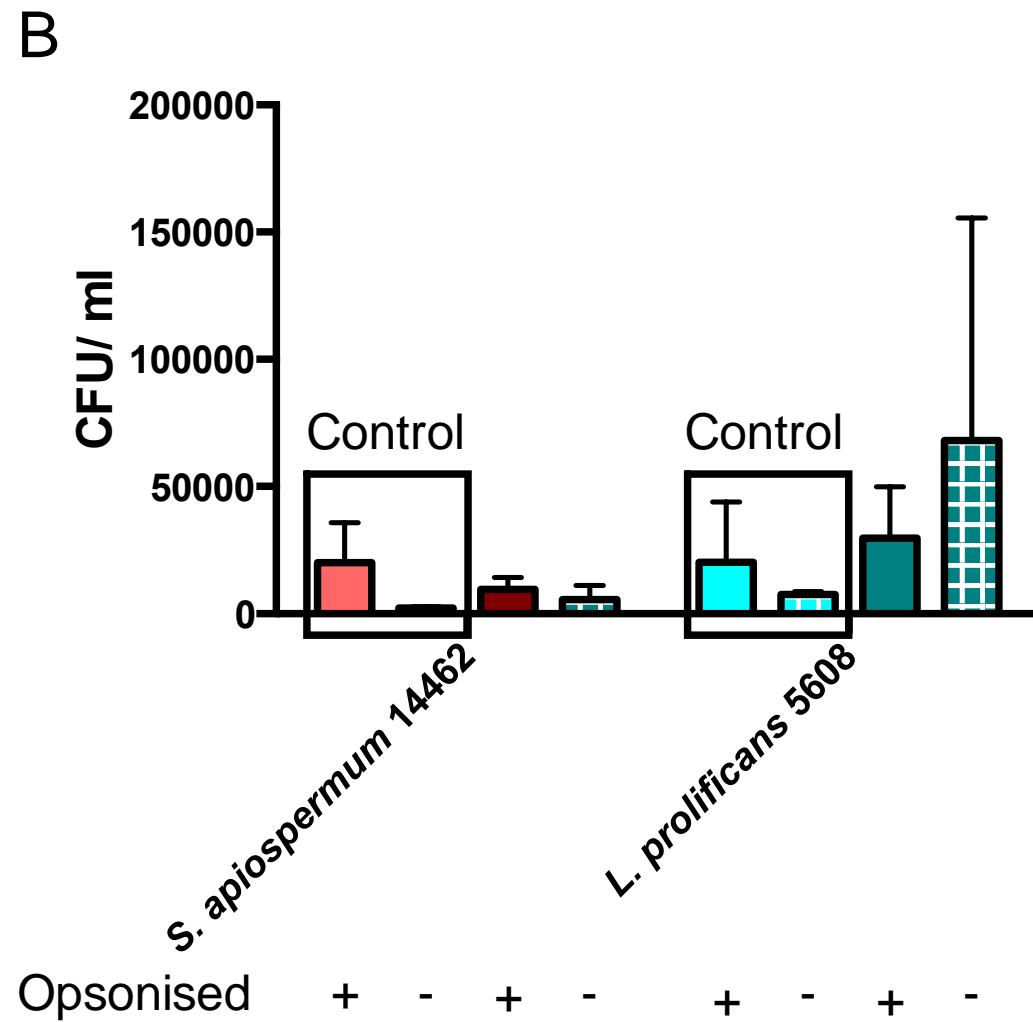
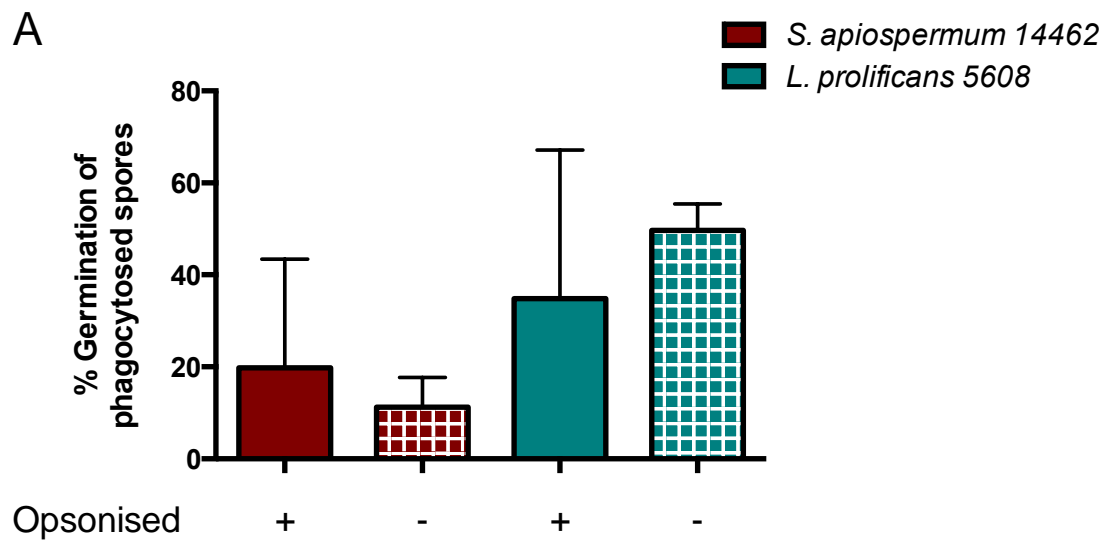
The J774A.1 murine cell line was infected with fungal spores of *Scedosporium* and *Lomentospora* spp. and time-lapse imaging was performed using LysoTracker Red (50 nM/ ml) to identify phagosome acidification with internalized spores. Movies were analysed and acidification was scored using NIS Elements software. Error bars represent standard deviation (n=3) statistical analysis was conducted using two-way ANOVA with Tukey's multiple comparison test. *p < 0.05, **p < 0.01, ***p < 0.00. There was no significant difference between strains.

***S. apiospermum* and *L. prolificans* spp. readily undergo lateral transfer, but do not exhibit vomocytosis, and are able to survive and germinate within macrophages despite serum opsonisation.**

It is known that for optimal phagocytosis, the presence of antibodies and complement components are required on the pathogen surface. It has been shown previously that serum opsonisation stimulates immune system and increase hyphae damage of filamentous fungi (Posch et al., 2017). Opsonisation of *Aspergillus* and *Cryptococcus* enhances phagocytosis, similarly to *Candida*, whose phagocytosis and killing is also enhanced by opsonisation (Shoham and Levitz, 2005). Therefore, we have investigated whether human AB serum opsonisation of *S. apiospermum* and *L. prolificans* spp. spores has any effect on germination within J774A.1 macrophages in comparison to unopsonised control (Figure 4.6 A); as well as the fungus survival within the murine macrophages followed by 2 hours infection (Figure 4.6 B). We found no significant difference between spores that has been opsonised and unopsonised in terms of germination and we confirmed these results by lysing infected cells and plating spores for CFU count (Figure 4.6 B). These results might suggest that complement receptors may not be involved in *Scedosporium/Lomentospora* spp. phagocytosis nor survival within macrophages. Although, more studies in this direction would have to be done to make a clear statement.

It is also known that some fungal pathogens are able to escape from host phagocytes by two processes, non-lytic expulsion (vomocytosis) and cell-cell transfer (lateral transfer). Therefore, we investigated whether *S. apiospermum* and *L. prolificans* spp. are able to vomocytose and/or undergo lateral transfer, and if human AB serum opsonisation makes any difference to those events in comparison to

unopsonised fungal spores (Figure 4.5 C). Intriguingly, both *Scedosporium* and *Lomentospora* spp. were able to lateral transfer but not undergo vomocytosis (Figure 4.5 C), making these the first intracellular pathogenic fungi known not to be vomocytosed.



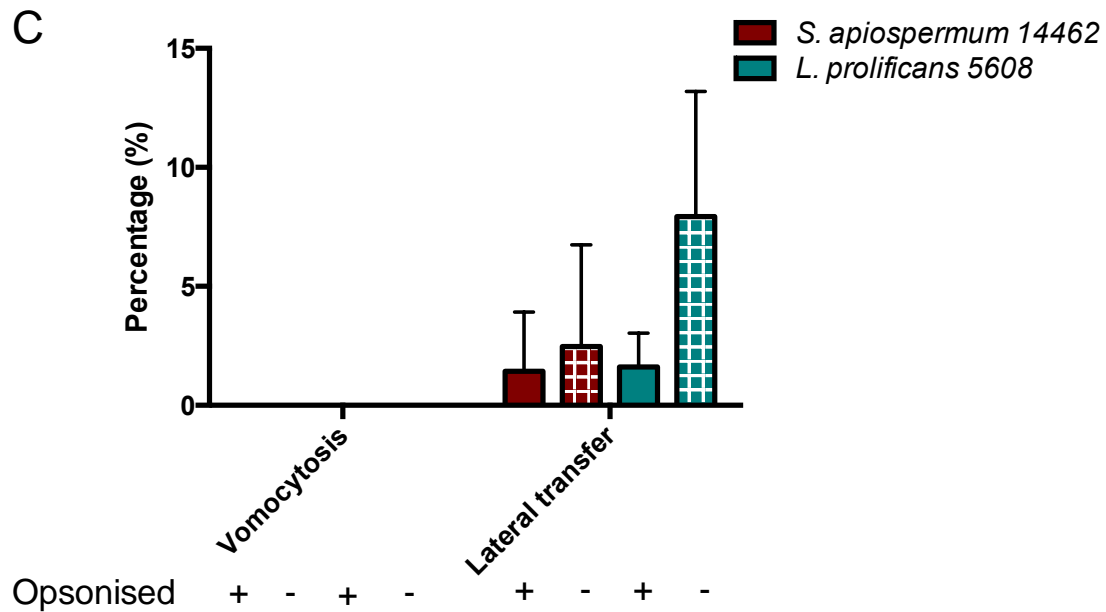


Figure 4.6 *Scedosporium* and *Lomentospora* spp. exhibit lateral transfer, but do not vomocytose, and survive within the host despite serum opsonisation.

J774A.1 murine cell line was infected with fungal spores of *Scedosporium* and *Lomentospora* spp. and time-lapse imaging was performed using a Nikon Eclipse Ti microscope with LWD 0.52 20x objective for 18 hours (A and C). Spore pellet was resuspended in serum free DMEM and some were opsonised with Human AB Serum prior infection of murine macrophage cell line J774A.1. Followed 2 hours infection, macrophages were lysed and spores were plated on potato glucose agar plates for CFU count. As a control, spores were also plated without phagocytosis. Agar plates were counted few days later (B).

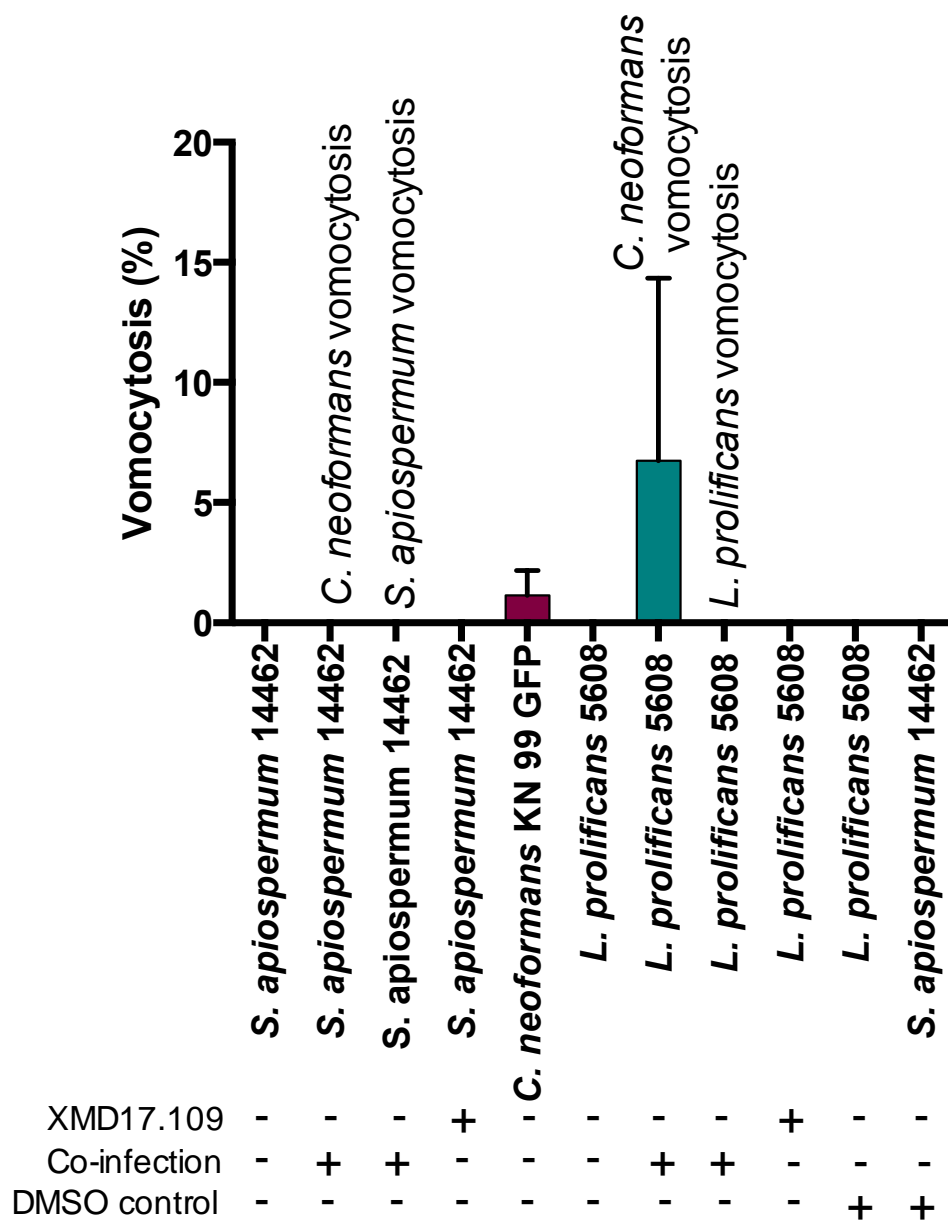
Movies were analysed and germination, lateral transfer and vomocytosis events percentage (%) was scored using NIS Elements software. Error bars represent standard deviation (n=3). Statistical analysis was conducted using one-way ANOVA with Tukey's multiple comparison test. *p < 0.05, **p < 0.01, ***p < 0.001 for graph (A,

B), and two-way ANOVA with Sidak's multiple comparison test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ for graph (C).

Scedosporium and Lomentospora spp. exhibit ability to lateral transfer but do not vomocytose despite different conditions tested.

Following our observation of *S. apiospermum* and *L. prolificans* ability of lateral transfer and lack of vomocytosis we decided to test whether vomocytosis of *Scedosporium/Lomentospora* spp. could be triggered either by XMD17.109, an inhibitor of the kinase ERK5 that has previously been shown to induce vomocytosis) or by co-infection with *C. neoformans* KN99. Our results indicate that *Scedosporium* and *Lomentospora* spp. remained unable to vomocytose in either situation (Figure 4.7 A) in the coinfection assays, although individual macrophages were able to vomocytose Cryptococci, co-infecting *Scedosporium* or *Lomentospora* spores were not expelled (Figure 4.7 A). In both cases, lateral transfer was unaffected (Figure 4.7 B).

A



B

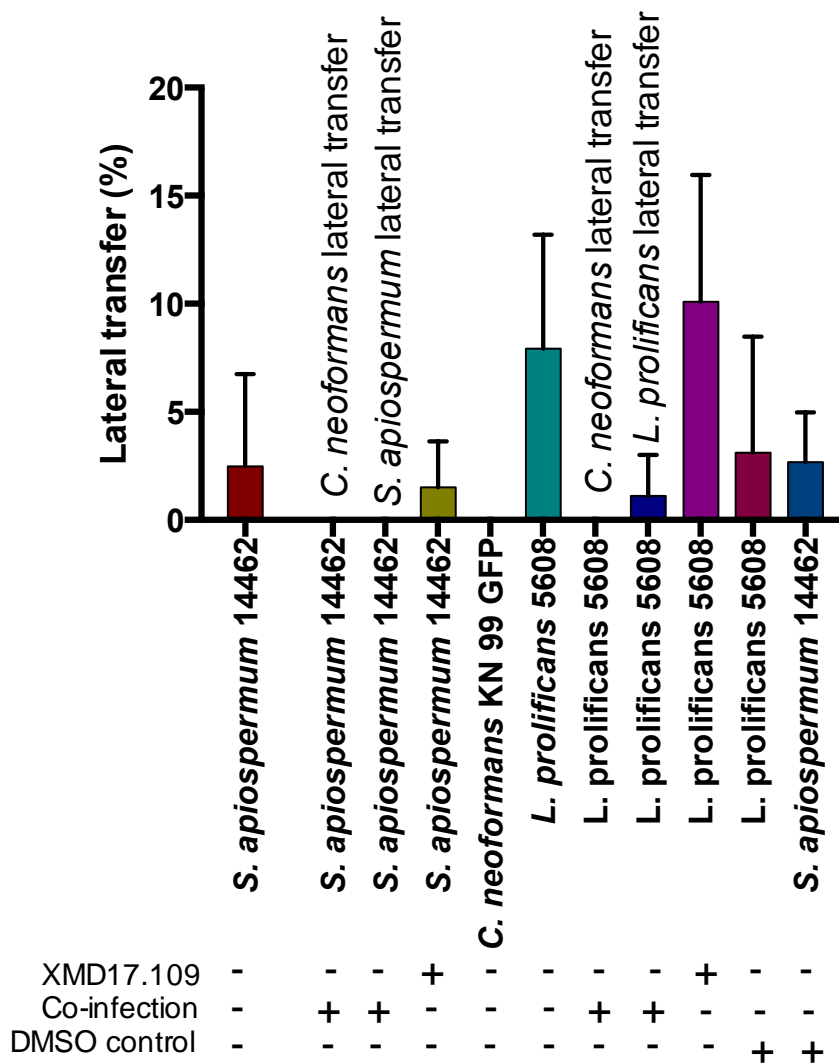


Figure 4.7 *Scedosporium* and *Lomentospora* spp. exhibit ability to lateral transfer but do not vomocytose despite different conditions tested.

The J774A.1 murine cell line was infected with fungal spores of *Scedosporium* and *Lomentospora* spp. and time-lapse imaging was performed using a Nikon Eclipse Ti microscope with LWD 0.52 20x objective for 18 hours. Movies were analysed and vomocytosis (A) and lateral transfer (B) events were scored using NIS Elements software. Error bars represent standard deviation (n=3). Statistical analysis was conducted using two-way ANOVA with Tukey's multiple comparison test. *p < 0.05, **p < 0.01, ***p < 0.001.

***S. apiospermum* and *L. prolificans* lateral transfer and lack of vomocytosis is conserved across different host cells.**

To test whether the remarkable behavior of *S. apiospermum* and *L. prolificans* is reproducible in macrophages from different backgrounds, we infected primary cells, Human Monocyte Derived- Macrophages (HMDMs) and iPSC-derived macrophages (Stem cells derived macrophages) cell line, and again scored for vomocytosis and lateral transfer. Our results indicate that lateral transfer and vomocytosis and germination pattern is conserved across different cell types tested (Figure 4.8-4.11). Those results for the first time present *Scedosporium* and *Lomentospora* spp. to be one of very few fungal pathogens that are not being able to undergoes vomocytosis and indicates that lateral transfer and vomocytosis are two separate events. We have also tested here, two strains kindly provided by Prof. Christopher Thornton (University of Exeter) such as *Lomentospora prolificans* albino (no melanin) mutant to WT 3.1 strain of *Lomentospora prolificans* Δ Lppks::hph. Al-Laaeiby et al. (2016) investigated how melanin of *L. prolificans* protects the fungus from H₂O₂ oxidative stress, UV and amphotericin B. They showed that melanin deficient strains are more susceptible to H₂O₂ oxidative stress and UV killing but not to amp B (Al-Laaeiby et al., 2016).

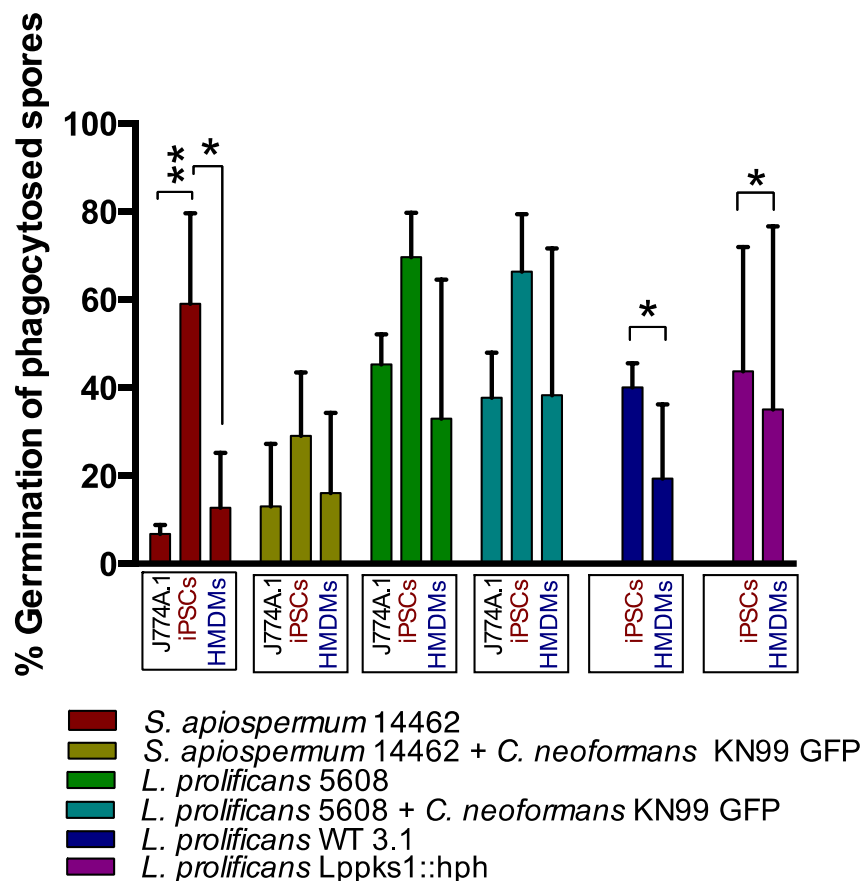


Figure 4.8 Germination of *Scedosporium* and *Lomentospora* spp. within macrophages remain conserved across different immune cell sources.

Murine macrophages cell line J774A.1, human monocyte derived macrophages (HMDM) and iPSC-derived macrophages were infected with *Scedosporium*, *Lomentospora* and *Cryptococcus* spp. and time-lapse imaging was performed using a Nikon Eclipse Ti microscope with LWD 0.52 20x objective for 18 hours. Movies were analysed and germination percentage (%) was scored using NIS Elements software. Error bars represent standard deviation (n=3). Statistical analysis was conducted using two-way ANOVA with Tukey's multiple comparison test. *p < 0.05, **p < 0.01, ***p < 0.001. We did not test *L. prolificans* WT 3.1 and Lppks::hph in infection with J774A.1 murine macrophages.

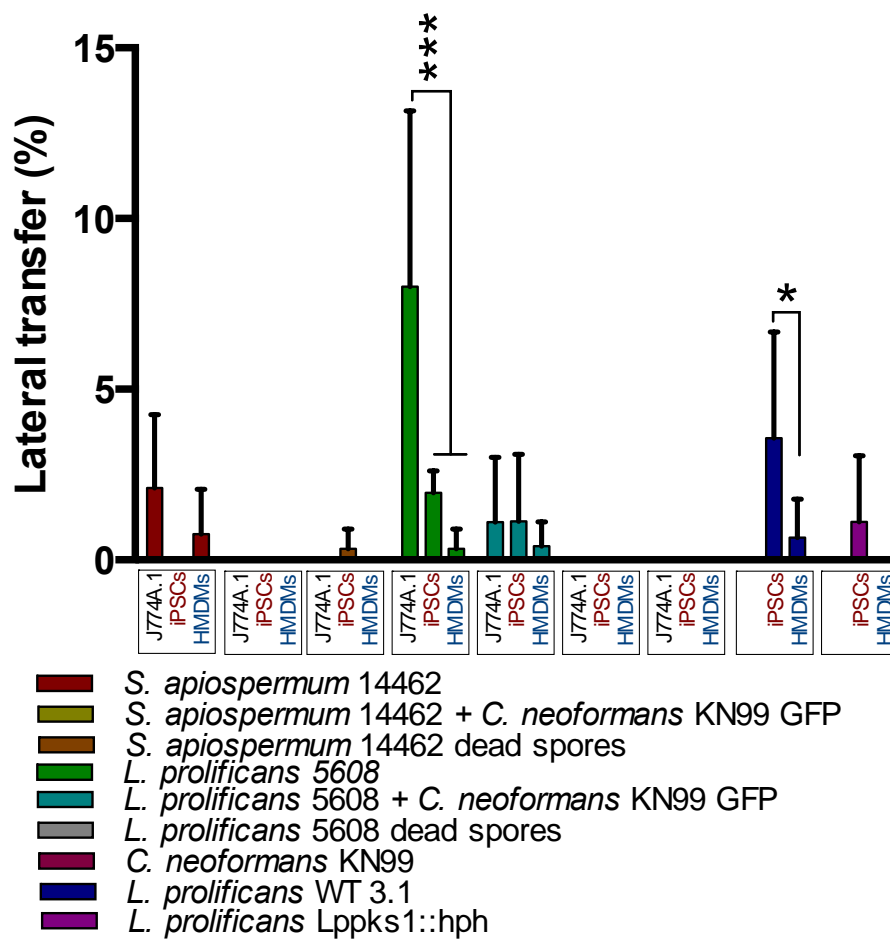


Figure 4.9 Lateral transfer was only noticed for *Scedosporium* and *Lomentospora* spp. but not for *Cryptococcus neoformans* across different immune cell sources.

The murine macrophage cell line J774A.1, human monocyte derived macrophages (HMDM) and iPSC-derived macrophages were infected with *Scedosporium*, *Lomentospora* and *Cryptococcus* spp. and time-lapse imaging was performed using a Nikon Eclipse Ti microscope with LWD 0.52 20x objective for 18 hours. Movies were analysed and lateral transfer percentage (%) was scored using NIS Elements software. Error bars represent standard deviation (n=3). Statistical analysis was conducted using two-way ANOVA with Tukey's multiple comparison test. *p < 0.05, **p < 0.01, ***p < 0.001.

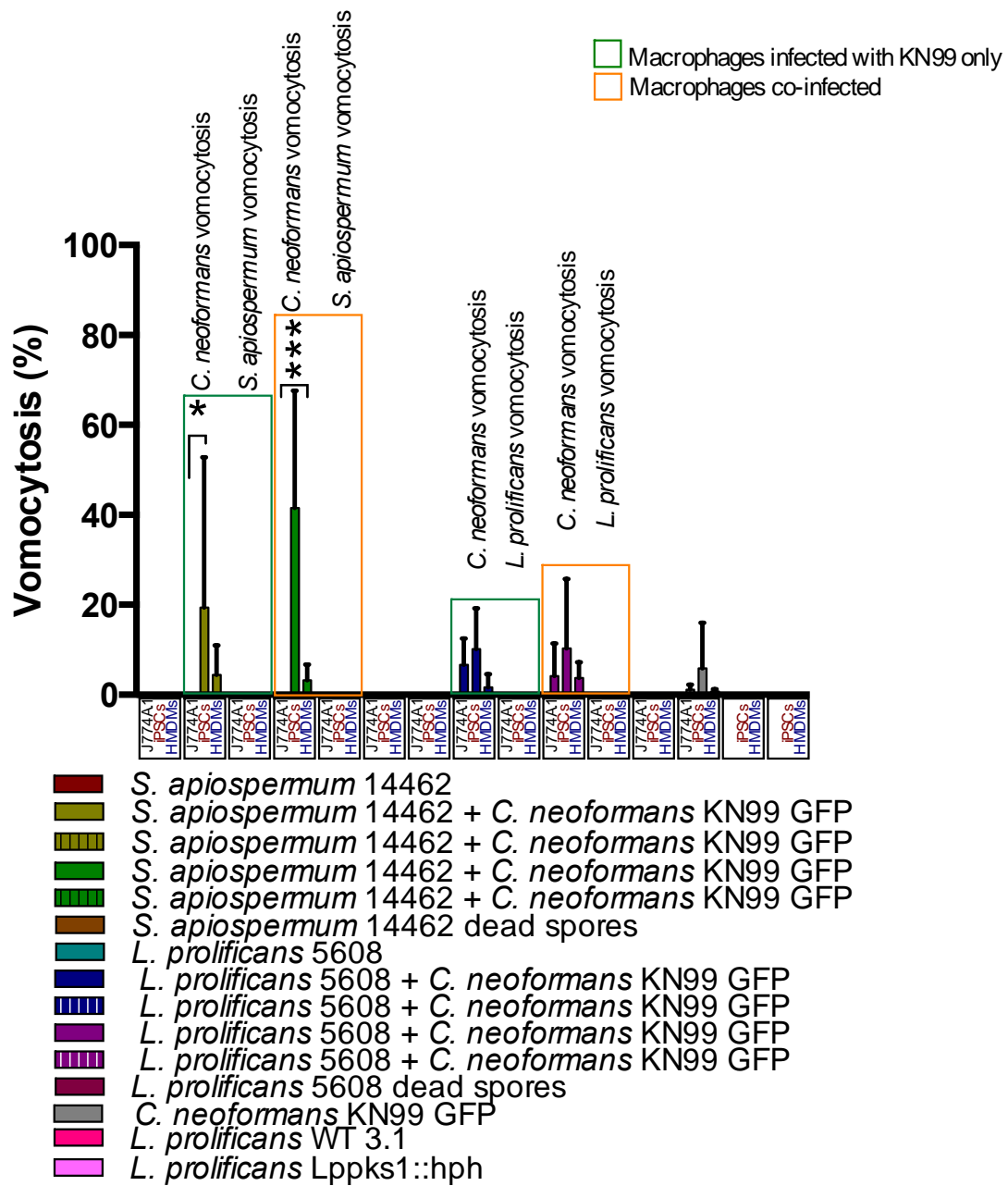


Figure 4.10 Vomocytosis was only recorded for *Cryptococcus neoformans* but not for *Scedosporium* and *Lomentospora* spp. across different cell sources.

Murine macrophages cell line J774A.1, human monocyte derived macrophages (HMDM) and iPSC-derived macrophages were infected with *Scedosporium*, *Lomentospora* and *Cryptococcus* spp. and time-lapse imaging was performed using a Nikon Eclipse Ti microscope with LWD 0.52 20x objective for 18 hours. Movies were analysed and vomocytosis percentage (%) was scored using NIS Elements software. Error bars represent standard deviation (n=3). Statistical analysis was conducted using two-way ANOVA with Tukey's multiple comparison test. *p < 0.05, **p < 0.01, ***p < 0.001. Percentage vomocytosis is the percentage of infected macrophages that has experiences at least one vomocytosis event.

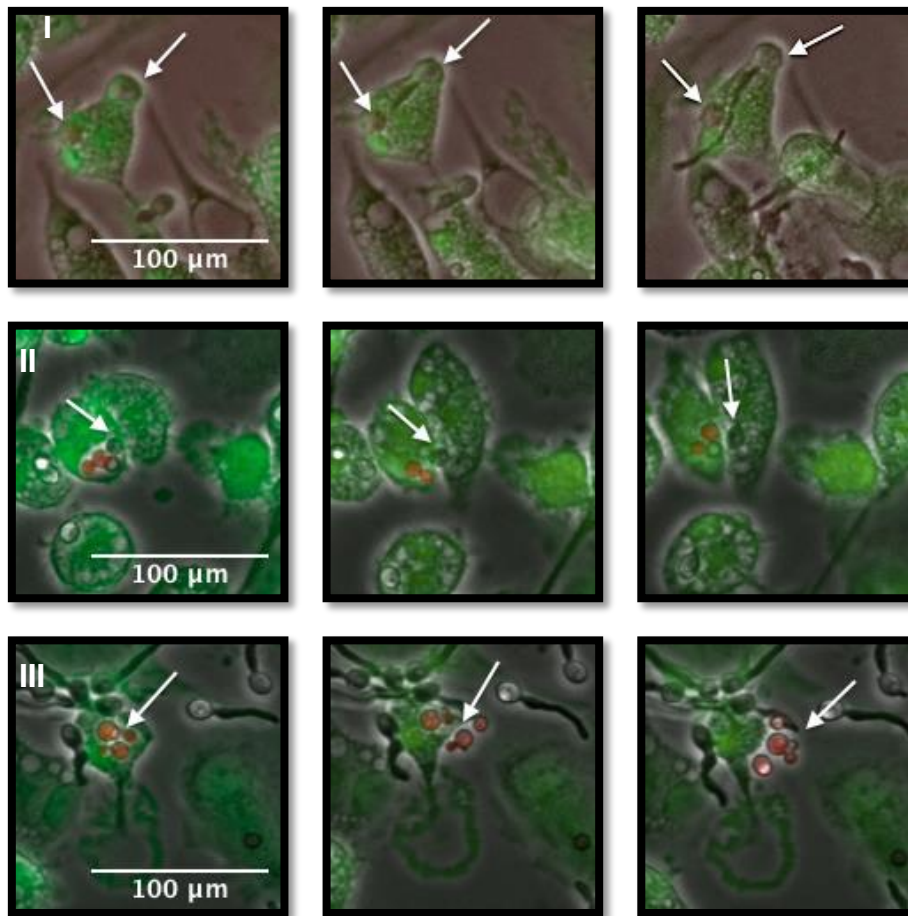


Figure 4.11 Visualisation of different behaviours of spores/cells of *L. prolificans* and *C. neoformans* KN99 (mCherry) within iPSC-derived macrophages.

iPSC-derived macrophages were infected with *Lomentospora* and *Cryptococcus* spp. and time-lapse imaging was performed using a Nikon Eclipse Ti microscope with LWD 0.52 20x objective for 18 hours. Movies were analysed using NIS Elements software.

I) Different spore behaviours of *L. prolificans* within a single macrophage. One spore never germinates, while the other germinates and penetrates the macrophage. II) An event of lateral transfer of *L. prolificans*. III) *C. neoformans* undergo vomocytosis (in red) leaving the macrophage alive, but not for long since the same macrophage is infected with *L. prolificans* (in black) that germinates within macrophage and will soon penetrate and kill the cell.

4.4 Discussion

Here we describe the interaction of *Scedosporium* and *Lomentospora* spp. with macrophages of the innate immune system, the first line defense for pathogenic fungi.

One of the first phagocytes approaching fungal pathogens are macrophages. They engulf and attempt to destroy and eradicate the pathogen. To do so, the macrophage phagosome undergoes maturation, and becomes acidified, highly oxidative and full of hydrolytic enzymes, which together kill the pathogen (Smith et al., 2015). Despite this remarkable ability of phagocytes to eradicate microbes, many fungal pathogens are known to be able to avoid killing (for example by manipulation of phagosome acidification) and/or escape from the cell via vomocytosis or lateral transfer (Gilbert et al., 2017; Shah et al., 2016).

We have measured the percentage of spores germinated inside innate immune cells causing their penetration and death. Our data showed that different macrophages tested, such as murine macrophages cell line J774A.1, human monocyte derived macrophages (HMDM) and iPSC-derived macrophages are not able to inhibit spore germination followed by phagocytosis. *S. apiospermum* remain less virulent than *L. prolificans* in terms of macrophage penetration and killing. These results are similar to those presented by Roilides et al. (2009) who have shown that HMDM's inhibited germination of *L. prolificans* less efficiently than *A. fumigatus* spores as a comparison. Al-Laaiby et al. (2016) investigated how melanin of *L. prolificans* strain, protects the fungus from H₂O₂ oxidative stress, UV and amphotericin B. They showed that melanin deficient strains are more susceptible to H₂O₂ oxidative stress and UV killing but not to amp B (Al-Laaiby et al., 2016). However, we showed that the melanin-deficient strains

(kindly provided by Prof. Christopher Thornton (University of Exeter)) remain virulent within macrophages and germinated, penetrating and killing them.

It is well known that coating fungal cell surfaces with complement proteins or opsonizing antibodies will enhance pathogen phagocytosis by macrophages (Erwig and Gow, 2016). However, we wanted to also investigate whether opsonisation of *Scedosporium* and *Lomentospora* spp. would have any effect on fungal survival within the murine macrophages cell line J774A.1. We found that there is no significant difference within opsonised and unopsonised spores in terms of fungus survival within the macrophage. This may be due to the fact that complement receptors are not involved in phagocytosis/survival of *Scedosporium* and *Lomentospora* spp. within murine macrophages, although this remains to be tested.

Phagosome acidification plays a key role in pathogen killing. The low pH within the phagosomal lumen is achieved by proton pumping of V-ATPases (Flannagan et al., 2012). Microbial growth is restricted by the acidic environment but it also enhances proteolytic enzyme activation (Flannagan et al., 2012). Acidification is a marker of a mature phagosome, therefore we used LysoTracker Red probes to detect acidification of the phagosome following phagocytosis of *Scedosporium* and *Lomentospora* spp. We found that live spores of both strains are not efficiently killed due to very low phagosome acidification in comparison to heat-killed spores. These results suggest that the pathogen is able to manipulate host cells to avoid getting killed by acidification. It is known that some pathogens are able to manipulate the acidic environment and survive, for instance, *Helicobacter pylori*, which is able to produce ammonium from urea to buffer the phagosomal pH, to prevent maturation and allow for intraphagosomal survival (Flannagan et al., 2012). Similarly, *Histoplasma capsulatum* inhibits

accumulation of V-ATPase, however, how it does so remains unclear (Flannagan et al., 2012). *Listeria monocytogenes* express LLO, a pore-forming toxin, that prevents phagosome maturation and perturbation of luminal H⁺ and Ca₂⁺ concentrations (Flannagan et al., 2012). However, the question of how *Scedosporium* and *Lomentospora* spp. remain viable within the phagosome due to the early study of those fungal spp. remains unanswered.

Another very interesting event is vomocytosis and/or lateral transfer. It is now known that some fungi are able to evade killing and escape from the phagocyte by two processes, non-lytic expulsion (vomocytosis) and cell-cell transfer (lateral transfer) (Ma et al., 2007; Gilbert et al., 2017; Ma et al., 2006; Shah et al., 2016). Therefore – we looked to see if these fungi are also able to do that. Our data using different fungal species suggest that vomocytosis and lateral transfer are two distinct processes. *Scedosporium* and *Lomentospora* spp. readily undergo lateral transfer, but are rarely vomocytosed, while the inverse was true for *Cryptococcus* spp. More interestingly, spores transferred to another macrophage were either killed or germinated inside the phagosome, leading to macrophage death. These results put *Scedosporium* and *Lomentospora* spp. in a unique position of being one of very few fungal pathogens studied that do not perform vomocytosis. Interestingly, even treatment of macrophages with XMD17.109 did not encourage either of the species to vomocytose. This suggests that vomocytosis host cell escape by *Scedosporium* and *Lomentospora* spp. are being controlled by a different mechanism than vomocytosis in *Cryptococcus*. Also, those two fungal pathogens exhibit interesting behaviour in comparison to *A. fumigatus* that is known to be able to lateral transfer in HMDMs. Shah et al., (2016) has demonstrated that lateral transfer of *A. fumigatus* is calcineurin orchestrated. This mechanism is

thought to control fungal burden by programmed necrosis-dependent spore lateral transfer between macrophages and inhibition of spore germination. However, my observations suggests that lateral transfer of both *Scedosporium* and *Lomentospora* spp. are controlled by a different mechanism as they are not associated with macrophage necrosis (before lateral transfer) nor with inhibition of spore germination. Therefore, this important biological question remains unanswered at the moment and requires further investigation to be able to understand the importance of lateral transfer in patients' outcome.

Taken together, *Scedosporium* and *Lomentospora* spp. behaviour within the innate immune cells observed in this study, such as prevention of phagosome acidification, ability to germinate within the macrophages, lateral transfer and lack of vomocytosis raise very important biological question of how these fungal species manipulate the host to survive and remain virulent. These unanswered biological questions may have an important impact on the medical condition of patients suffering from scedosporiosis and need to be investigated further.

5. DEVELOPING AN *EX-VIVO* SKIN MODEL AS A NOVEL APPROACH TO INVESTIGATE PATHOGENICITY OF FUNGAL SPORES

Research Aim

Investigate immune cells isolated from the *ex-vivo* skin interaction with fungal pathogens.

5.1 Introduction

Skin, the biggest organ of the human body, is permanently exposed to the environment (Merad et al., 2008). Skin provides the first line of defense to all microbial pathogens, both those found in the environment as well as commensal organisms. By breaching this barrier, pathogens are able to get inside the body and cause life-threatening infections. Consequently, it is very important to understand the role of skin as a barrier that protects us from environmental contamination and its role in immune defence. Immune responses in the skin is the focus in this chapter of research.

Skin can be divided into two major layers, the epidermis and dermis (Kaplan., 2010). Epidermis is derived from ectoderm, mainly composed of keratinocytes and Langerhans cells (LCs), which are the only dendritic cells found in this layer during steady state (no inflammation) (Kaplan., 2010). In contrast, dermis is a mesoderm-derived layer, mainly populated by fibroblasts, memory T cells, mast cells and dendritic cells (Kaplan., 2010) (Figure 5.1).

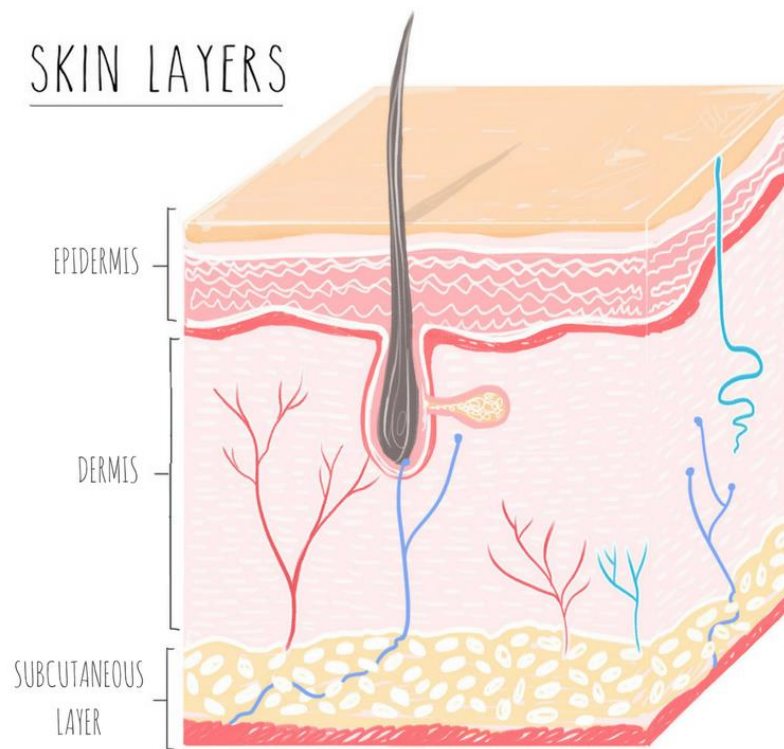


Figure 5.1 Human skin layers.

Figure adapted from DermStore.com.2015 <https://www.dermstore.com/blog/layers-and-functions-of-skin/>

Langerhans cells

LCs are antigen presenting cells, able to detect pathogens and then mature, presenting peptide antigens to T cells in the lymph nodes (Henri et al., 2010). LCs survey the epidermal surface with their extended dendrites to search for foreign pathogens (Merad et al., 2008). Earlier work suggested that CD207 (langerin) was a definitive marker for LCs (Merad et al., 2008). However, recent studies have shown there are a number of LC subsets, including CD207⁺DCs in steady state in central lymph nodes, CD207^{low} CD8 α ⁺ DCs found in lymphoid tissue and CD11c^{inter-to-high} MHCII^{high} phenotype CD207⁺, originating from skin (Henri et al., 2010). A unique future

of LCs are Birbeck granules, identified in 1961, that are intracytoplasmic organelles thought to have antimicrobial properties (Merad et al., 2008). Their function is still a debate; however, they are thought to be migrating to the periphery of the LCs and releasing their contents to the extracellular matrix and/or function as a receptor-mediated endocytosis (Mc Dermott et al., 2002; Kissenpfennig et al., 2005). Langerhans cells express various markers on their surface, such as E-cadherin (CDH1), epithelial-cell adhesion molecule (EpCAM), CD205 and CD1a. These are responsible for attaching LCs to neighbouring keratinocytes, adhesion, antigen capture and processing and antigen presentation to T cells, respectively. Langerhans cells are a type of tissue-resident macrophage (Perdiguero and Geissmann., 2016). Epidermal LCs have the ability to self-renew and are also maintained by a pool of radioresistant proliferating host haematopoietic precursor cells that reside in the skin. Repopulation of Langerhans cells in the skin during steady state (without inflammation) occurs very slowly in comparison to DC repopulation in the lymph nodes or spleen. However, during inflammation (active state), LCs are repopulated by circulating monocytes through an M-CSF- dependent pathway (Merad et al., 2008). The LC maturation process is not well understood. LCs that recognise microbial antigens secrete pro-inflammatory cytokines, triggering a wider immune response. According to Merad et al. (2008), different methods of isolating LCs (LCs from mouse or human skin isolated using enzymatic digestion of skin, or short-term culture of skin pieces) may have a different effect on gene expression of Langerhans cells, thus making it difficult to study DC maturation. Different gene expression might affect LCs maturation, therefore it can dramatically affect the expression of surface markers and consequently their response to pathogens. *In vitro* studies have shown that LCs isolated from human skin can

efficiently activate T helper 1 cells in response to antigen recognition *in vitro*. They can also cross-present (antigen present) them to CD8⁺ T cells (Merad et al., 2008).

Dermal dendritic cells

Two types of myeloid DCs, spontaneously migrating from skin *in situ* samples have shown overlapping profile of CD14⁺ DCs with dermal macrophages (Haniffa et al., 2015). However, these can be then distinguished by morphology, flow cytometry, migratory behaviour, adherence and turnover kinetics. Dermal dendritic cells (dDC) in skin play a key role in inflammatory skin diseases. There are two types of dermal DC cells that are normally present in the skin, those that reside in skin during the steady state and those that are recruited during inflammation, they have a different marker expression on their surface (Haniffa et al., 2015). The distribution of human dendritic cells, monocytes and macrophages in skin, blood and lymph nodes are shown below (Haniffa et al., 2015).

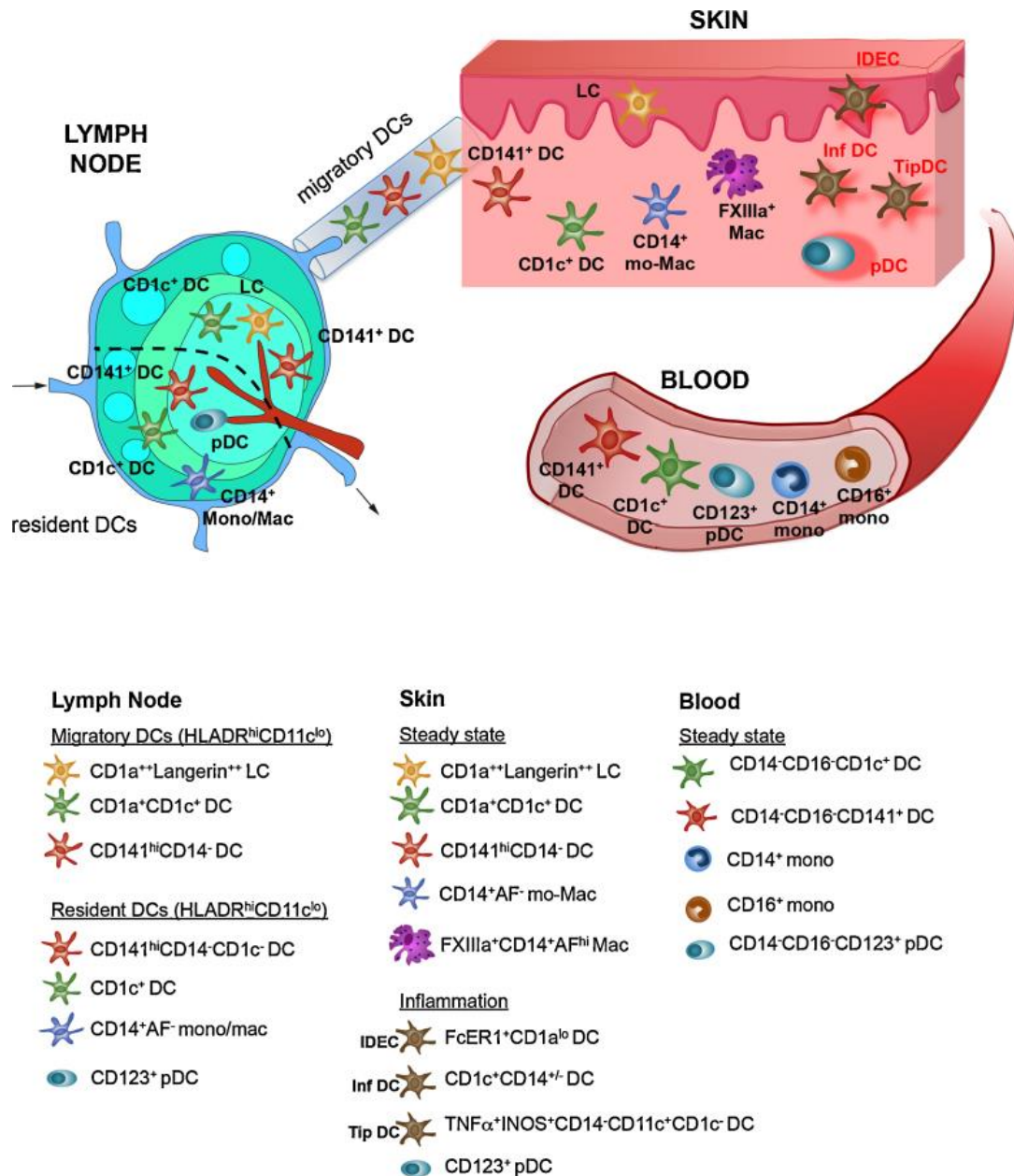


Figure 5.2 Distribution of human dendritic cells, monocytes and macrophages in skin, blood and lymph nodes.

Changes during inflammation are indicated in red text. pDC = plasmacytoid DCs, Mac = macrophage, mono = monocytes, mo-Mac = monocyte-derived macrophage, inf DC = inflammatory DCs, IDEC = inflammatory dendritic epidermal cells, TipDC = TNF α and iNOS producing DC. Adapted from Haniffa et al., 2015.

DCs produce TNF α and iNOS in response to bacterial pathogens (Haniffa et al., 2015). The mechanism that controls langerin⁺ cells activation in skin is not known (Merad, et al., 2008).

Recent studies have been focused on targeting DCs as a major route of vaccination (Macri et al., 2016). Macri et al. (2016) have attempted to understand the complexity of the different subsets of DCs to design novel vaccinations based on an antigen-conjugated antibody strategy. As the current vaccines are composed of pathogen-derived antigens that give rise to antigen-specific memory of B and T cells, the DCs (antigen presenting cells), becomes an interesting vaccination target (Macri et al., 2016).

Chu et al. (2009) have investigated immunoregulatory potential of dermal DC and showed that they possess capacity of migration to lymph nodes. Moreover, those cells induce regulatory T cells that inhibit skin inflammation. This group has also shown, that vitamin D₃ up regulated expression of CD141 marker (found on dermal DC) in DC derived from blood (that are CD141 negative). They suggest that, this finding might be of use to generate cells *in vitro*, and once transferred *in vivo*, cells will have a capacity of xeno-graft versus host disease and tumor alloimmunity (Chu et al., 2009).

Schaeffer, E. et al. (2015) have investigated how dDC impact on the immune response to Dengue virus (DENV), using cells isolated from skin tissue by 'walk-out'. Walk-out methods rely on cells leaving the tissue suspended in media. Their findings have shown they were unable to detect TNF α production by dDCs in response to DENV in comparison to other research (Haniffa et al., 2012) that shown production of TNF α by CD1c⁺ and CD14⁺ dermal DC.

Here we are investigating if *ex-vivo* skin tissue sections could provide a suitable model for the study of fungal wound infections. Fungal species discussed in this thesis are found in the environment. Following traumatic injury, fungal spores are easily being introduced into the wound and germinate and spread. The immunology of skin resident cells and its interaction with microbes are poorly understood. Therefore, we have an taken approach, previously described by some research groups (Haniffa et al., 2015; Schaeffer, E. et al., 2015) to investigate interaction of LCs/dDCs, isolated from epidermis and dermis *ex-vivo* skin tissue, with fungal pathogens such as *Mucormycete* and *Scedosporium* spp.

5.2 Materials and Methods

Unless otherwise stated all reagents were purchased from Sigma Aldrich UK.

Strains and growth condition. Strains used in experiments were *Rhizopus microsporus* 12.6652333 (clinical isolate) and *Scedosporium apiospermum* IHEM 14462. *R. microsporus* strain was grown on Sabouraud agar plates (VWR) for at least 10 days before use. *S. apiospermum* strain was grown on Potato Glucose agar plates for at least 10 days before use. For infection, spore colonies of *S. apiospermum* 14462 and *R. microsporus* were harvested from 2-week-old potato glucose/ sabouraud agar (VWR), using PBS and centrifuged at 1000 x g for 3min, maximum breaking and acceleration. Spores pellet was resuspended in PBS for counting on haemocytometer.

Skin cell isolation from skin off-cuts. Skin samples generated during surgical procedures were registered and released via the Human Biomaterials Resource Centre (HBRC) in Queen Elizabeth Hospital, Birmingham and then transported in DMEM + 100 U/ml of Penicillin- Streptomycin solution on ice (within 1 hour of skin off cut) (Figure 5.3 A). After removal of cutaneous fat tissue (by sterile scalpel) (Figure 5.3 B), 300 µm of epidermis and dermis layers were removed by dermatome (Nouvag) following the manufacturers instructions (Figure 5.3 C). Skin layers were cut into 1cm square pieces (Figure 5.3 D) and incubated in an enzyme cocktail (1 mg/ml of collagenase A (Thermo Fisher Scientific); 0.05 mg/ml of Deoxyribonuclease I from bovine pancreas DN25; 2.5 mg/ml of Dispase II, (Thermo Fisher Scientific); RPMI 1640 (Thermo Fisher Scientific) 10 µl/ml of Penicillin- Streptomycin solution) for 30 min at 37° C shaking water bath. Then the epidermis layer was mechanically separated from the dermis layer (Figure 5.3 E) and incubated in RPMI1640 (Thermo Fisher Scientific) supplemented with 100 U/ml of Penicillin- Streptomycin solution and Human

serum from human male AB plasma 10 % for 48 hours in 37° C, humidified, 5% CO₂, to allow cells to leave the tissue (Figure 5.3 F).

Figure 5.3 A

Skin sample was transported in DMEM + Pen/Strep solution on ice within 1 hour of skin off cut.



Figure 5.3 B

Removal of cutaneous fat tissue.

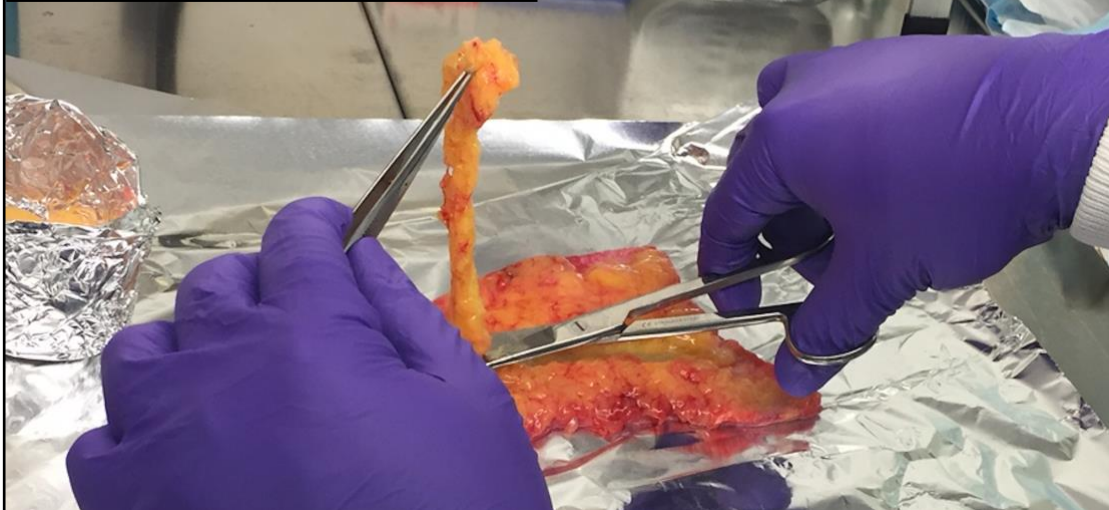


Figure 5.3 C

300 μ m of epidermis and dermis layers were removed by dermatome.



Figure 5.3 D

Skin layers were cut into 1 cm square pieces.

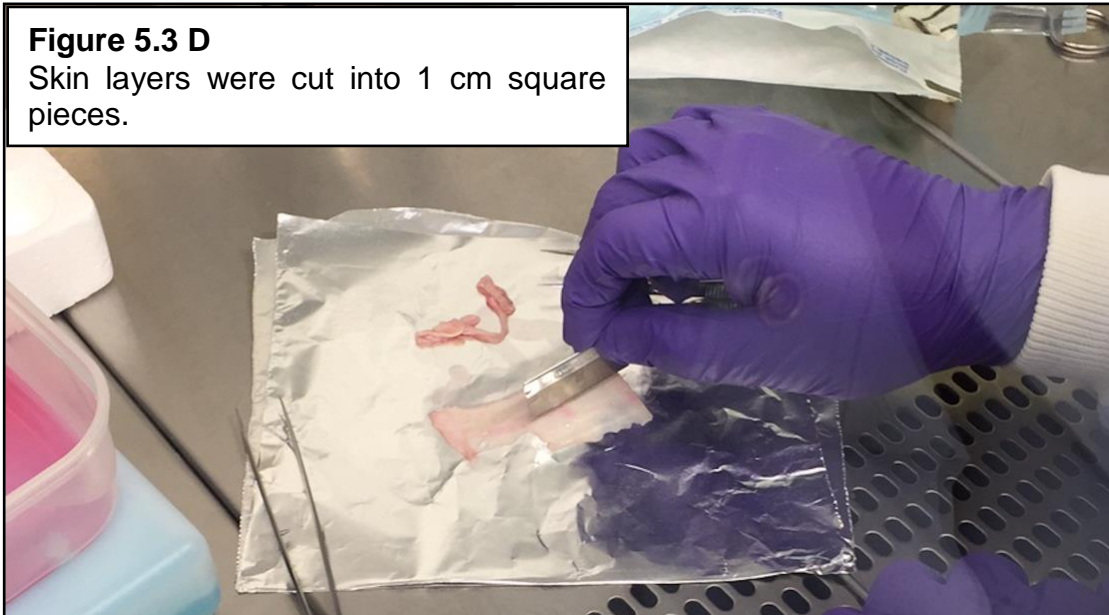


Figure 5.3 E

The epidermis layer was mechanically separated from the dermis layer.

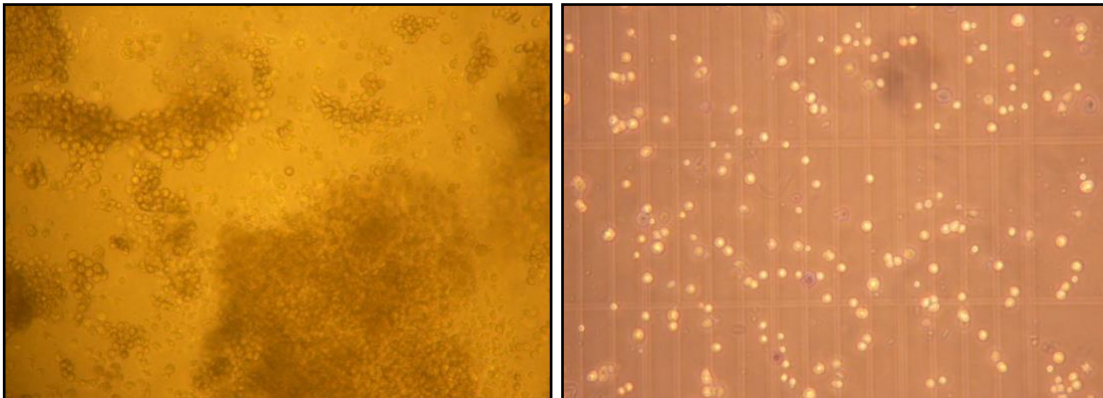
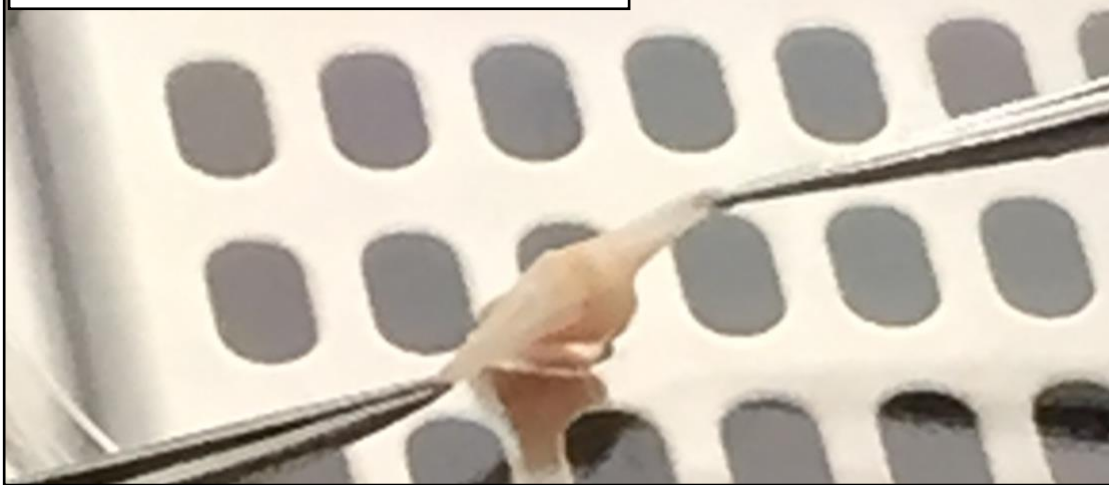


Figure 5.3 F

Skin cell that left tissue (left) and followed by centrifugation (right).

Figure 5.3 Visualisation of the methodology of steps in skin cells isolation from skin off-cut.

Skin cell infection with fungal spores and analysis. After 48 hours cells were collected from media and centrifuged and then infected with either dead or live spores of *Rhizopus microsporus* 12.6652333 or *Scedosporium apiospermum* IHEM 14462 (MOI 10:1) for 5 hours. After 1 hour of infection, 10 ug/ml of Brefeldin A (New England Biolabs) was added to samples to stop cytokine release into the media (to enable internal markers for TNF-alpha and CD83 detection following infection). After infection,

cells were washed with PBS and 10 % heat inactivated rabbit serum (MP Biomedicals) was added to cells on ice for 15 min for blocking. Following that, cells were first stained for extracellular markers with BV605 mouse anti-human CD11c (BD), PE mouse anti-human CD45 (BD), PerCP-Cy 5.5 mouse anti-human CD14 (BD), BV421 mouse anti-human CD141 (BD), BV510 mouse anti-human HLA-DR (BD), PE mouse anti-human Langerin CD207 (BD) (in concentrations suggested by BD supplier, all isotypes IgG) antibodies on ice in dark. Following that, cells were washed 3 times in PBS and fixed with 1% paraformaldehyde for 10 min in room temperature. Cells were washed with PBS then permeabilised with perm/wash buffer (BD) and stained for intracellular markers with FITC-conjugated mouse anti-human TNF-alpha (BD) or FITC-conjugated mouse anti-human CD83 (BD) antibodies, on ice in dark for 1 hour. Cells were then washed 3 times with PBS and analysed by flow cytometry with on an Attune Classic instrument with Auto Sampler (Applied Biosystems) to quantify cytokine production.

Flow Cytometry Panels – Dermal Cells

CD83 Panel		TNF- α Panel		Isotype Control Panel	
Marker	Fluorochrome	Marker	Fluorochrome	Marker	Fluorochrome
CD83	FITC	TNF- α	FITC	Isotype	FITC
CD45	PE	CD45	PE	Isotype	PE
HLA-DR	APC	HLA-DR	APC	Isotype	APC
CD11c	APC-Cy7	CD11c	APC-Cy7	Isotype	APC-Cy7
CD141	VioBlue	CD141	VioBlue	Isotype	VioBlue
CD14	PerCP	CD14	PerCP	Isotype	PerCP

Table 5.1 Mature DC panel.

Expression of CD83 is a hallmark of DC maturity, can be induced by pro-inflammatory cytokines, LPS, CD40 ligands; TNF α cytokine released by keratinocytes play a key role in LC migration from epidermis, through dermis to the draining lymph nodes. CD45 is a marker of hematopoietic cells; HLA-DR is a marker of human leukocyte antigen complex; CD11c is a marker of dermal dendritic cells; CD141 is a marker of vascular endothelial cells; CD14 is a marker of dermal dendritic cells. All isotypes controls are IgG. Flow Cytometry Panel prepared by Dr. Matt Ivory (Cardiff University). Markers explanation by Wioleta Trzaska.

Flow Cytometry Panels – Epidermal Cells

CD83 Panel		TNF- α Panel		Isotype Control Panel	
Marker	Fluorochrome	Marker	Fluorochrome	Marker	Fluorochrome
CD83	FITC	TNF- α	FITC	Isotype	FITC
CD207	PE	CD207	PE	Isotype	PE
HLA-DR	APC	HLA-DR	APC	Isotype	APC
				Isotype	APC-Cy7
				Isotype	VioBlue
				Isotype	PerCP

Table 5.2 Mature LC panel.

Expression of CD83 is a hallmark of DC maturity, can be induced by pro-inflammatory cytokines, LPS, CD40 ligands; TNF α cytokine released by keratinocytes play a key role in LC migration from epidermis, through dermis to the draining lymph nodes. HLA-DR is a marker of human leukocyte antigen complex; CD207 is a marker of Langerhans cells. All Isotype controls are IgG. Flow Cytometry Panel prepared by Dr. Matt Ivory (Cardiff University). Markers explanation by Wioleta Trzaska.

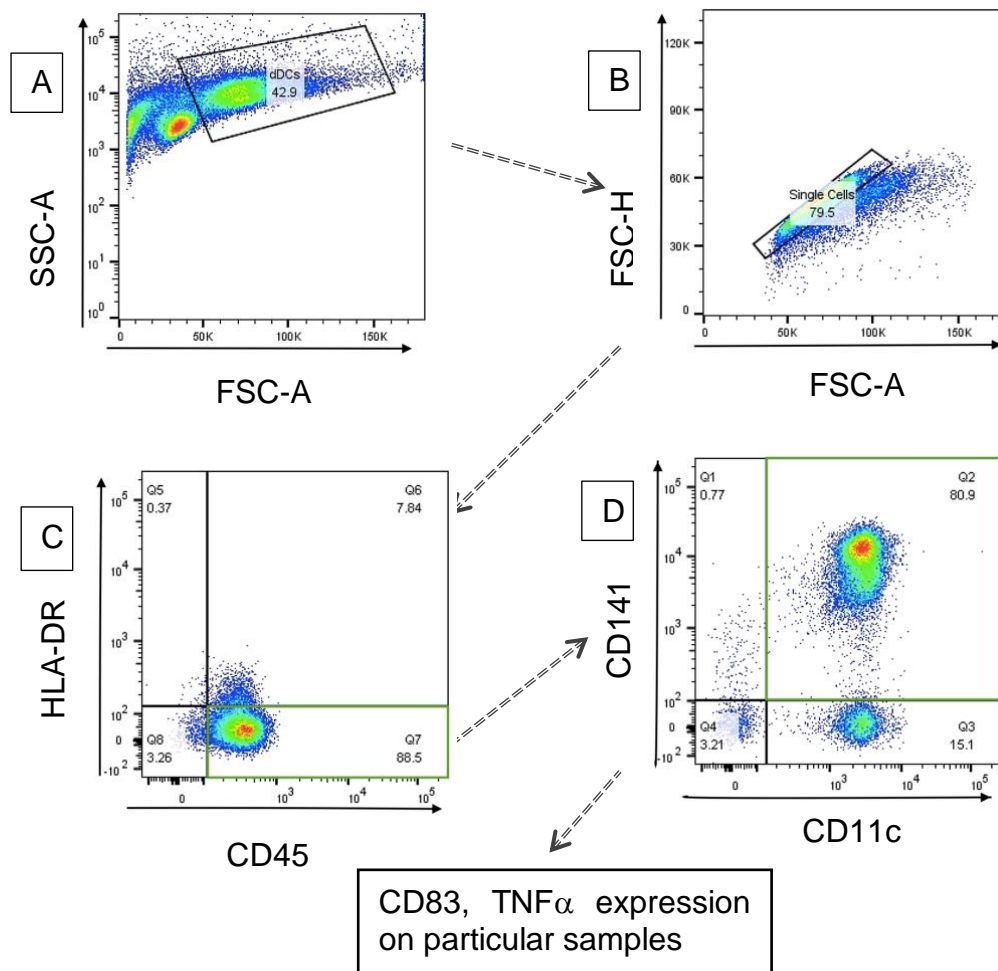


Figure 5.4 Example of flow-cytometry gating on cells isolated from ex-vivo skin sheets. First, cells are gated on FSC-A (Forward Scatter Area) /SSC-A (Side Scatter Area) for cell size and granularity (A). When cells pass through the laser beams, it scatters light at all angles (FSC and SSC). FSC is proportional to the cell size, while SSC is proportional to the internal complexity of the cell. Signals sent by cells are being detected as a voltage pulse which is processed and defined by its area (A), height (H) and width (W). Height is the intensity of the signal, while area is the height + width; therefore, the single cells were gated on FSC-H/FSC-A to separate singlets from doublets and clumps (B). Then the gating was on CD45/HLA-DR positive cells to include total dermal

DCs (C). Following that, cells were gated on CD141/CD11c (D), where cells of our interest were CD141 negative and CD11c positive. Next, cells were analyzed for CD83 or/and TNF α expression (results shown below).

5.3 Results

Langerhans cells do not undergo maturation following *R. microsporus* and *S. apiospermum* infection.

Skin is a barrier that protects us from environmental pathogens. It consists of three layers: epidermis, dermis and fat. Langerhans cells (LCs) account for 3-5 % of all nucleated cells in epidermis and their primary role is to detect pathogens, at which point they mature and migrate to skin-draining lymph nodes to present antigen to T-cells.

To investigate whether LCs undergo maturation and release of cytokines after contact with the human pathogens *R. microsporus* and *S. apiospermum*, we isolated LCs from epidermis and exposed them to spores of both fungal strains. However, upon fungal exposure, LCs neither increased expression of CD83 (a well-recognised marker of matured dendritic cells (Milne et al., 2015) nor expressed TNF α (a cytokine typically released by LCs to stimulate T cells). Thus, these preliminary data suggest that LCs may not be appropriately triggered to mature and present antigen following contact with either of these fungal pathogens ((Figures 5.5 (a, b); 5.6 (a, b); 5.7 (a)). It is important, however, to emphasize here that the very limited amount of skin samples available, as well as the early stage of this research, mean that these results will need independent verification in the future.

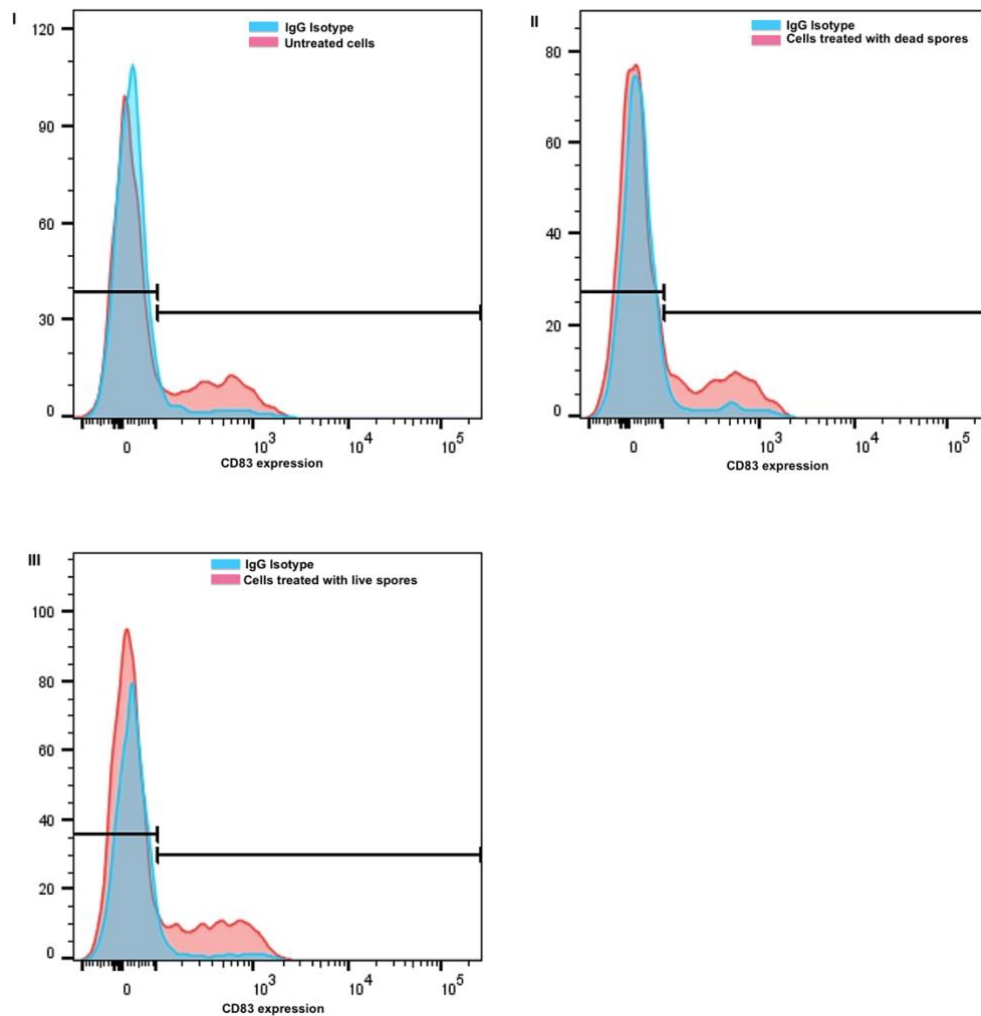


Figure 5.5 (a) CD83 expression of epidermal cells from “431” skin sample.

Histograms present untreated cells (I), cells treated with dead spores of *R. microsporus* (II), cells treated with live spores of *R. microsporus* (II). There is very little maturation of Langerhans cells basing on CD83 expression. This figure was analysed by Matt Ivory (Cardiff University).

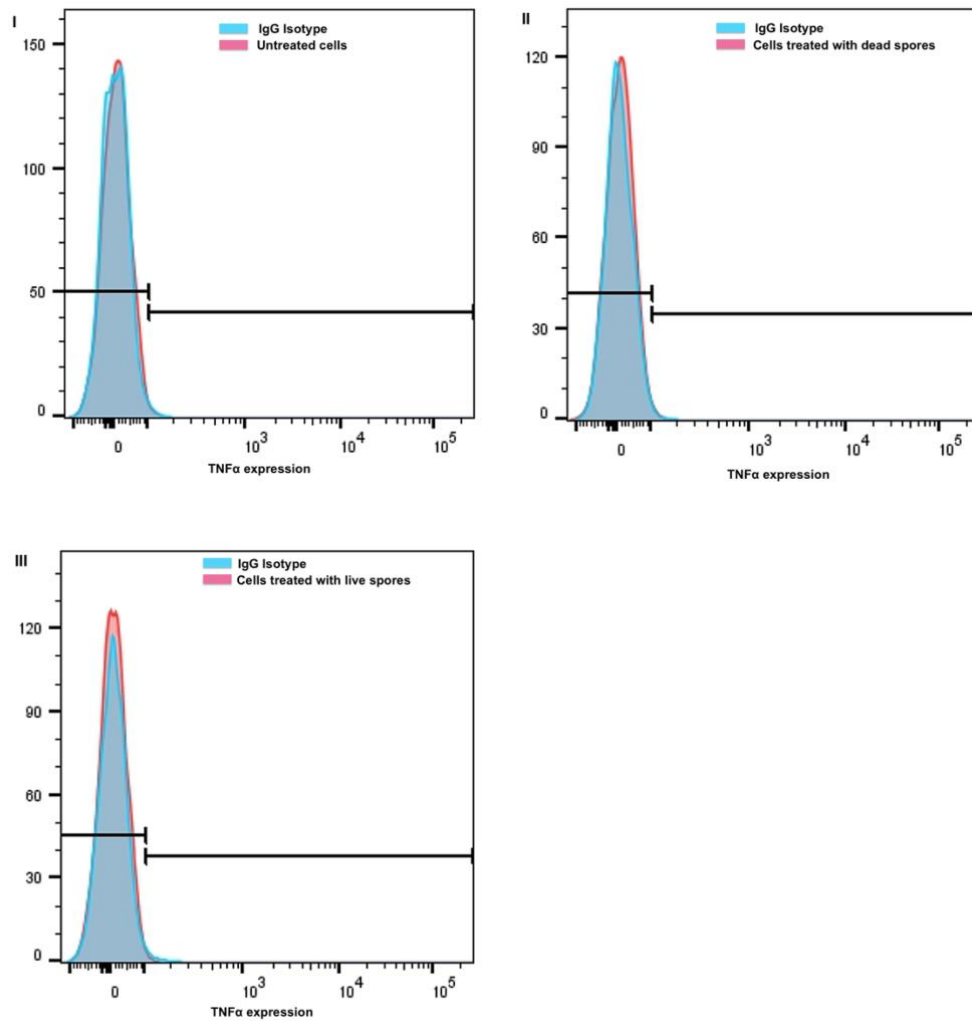


Figure 5.5 (b) TNFα expression of epidermal cells from “431” skin sample.

Histograms present untreated cells (I), cells treated with dead spores of *R. microsporus* (II), and cells treated with live spores of *R. microsporus* (III). There is no TNFα expression at all to fungal pathogen. This figure was analysed by Matt Ivory (Cardiff University).

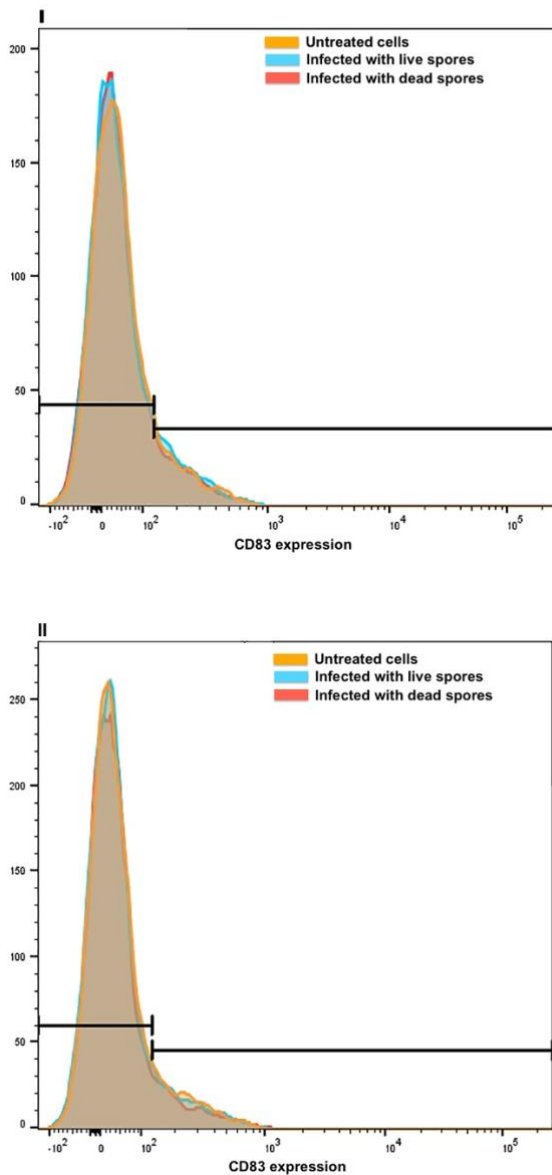


Figure 5.6 (a) CD83 expression of epidermal cells from “434” (I) and “435” (II) skin samples.

There is no increased CD83 expression in either of the sample following infection with spores of *R. microsporus*. This figure was analysed by Matt Ivory (Cardiff University). Both, Wioleta Trzaska and Matt Ivory undertook this experimental procedure.

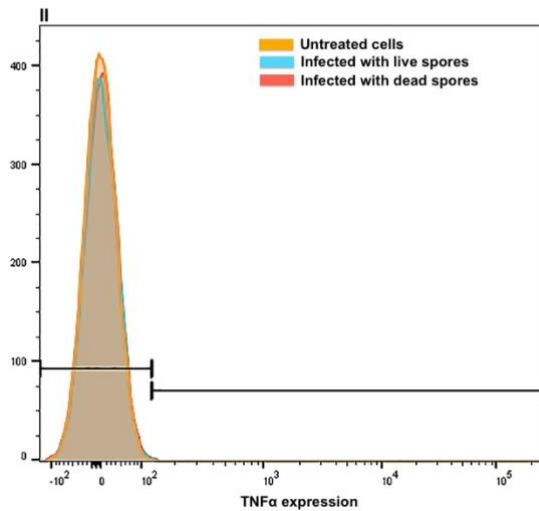
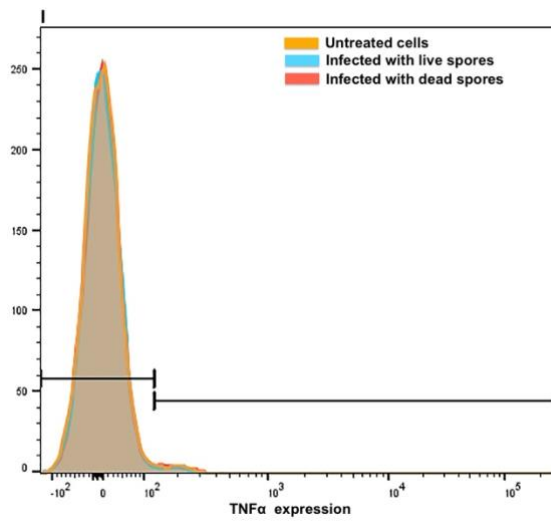


Figure 5.6 (b) TNF α expression of epidermal cells from “434” (I) and “435” (II) skin samples.

There is no TNF α expression in Langerhans cells following infection with spores of *R. microsporus* in either of the samples. This figure was analysed by Matt Ivory (Cardiff University). Both, Wioleta Trzaska and Matt Ivory undertook this experimental procedure.

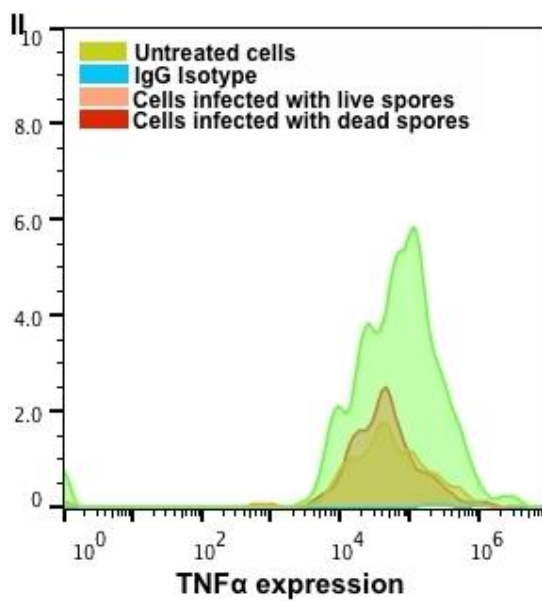
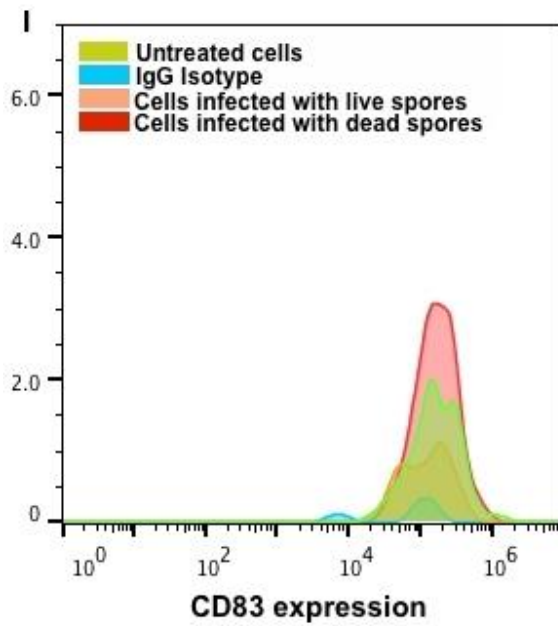


Figure 5.7 (a) CD83 and TNFα expression of epidermal cells from “001” skin sample.

There is no epidermal cells recognizable maturation CD83 (I) and no TNFα (II) expression following infection with spores of *S. apiospermum* 14462.

Dermal dendritic cells (dDC) undergo maturation followed by contact with *R. microsporus*, but do not release TNF α .

Destruction of skin due to an injury provides a route of entry for environmental pathogens such as *R. microsporus* or *S. apiospermum*. Following exposure to these pathogens, we observed that dDCs undergoes maturation (expressing CD83) in response to *R. microspores* but not *S. apiospermum*. Despite this, however, these activated dDCs did not produce TNF α ((Figures 5.8 (a, b); 5.9 (a, b); 5.7 (b)). Again, however, further experiments need to be performed to investigate these observations in more detail and, importantly, in other samples.

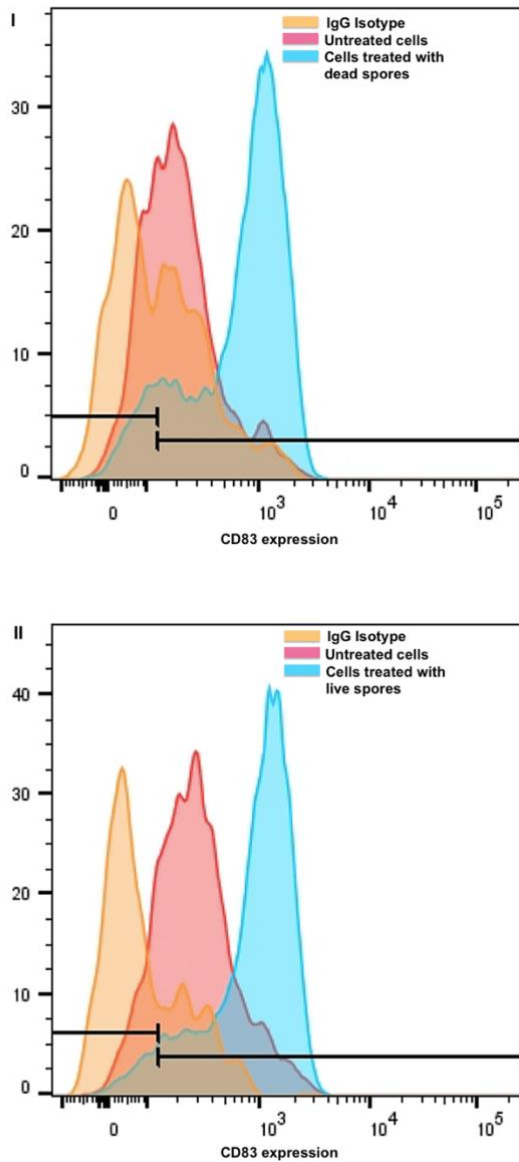


Figure 5.8 (a) CD83 expression of dermal cells from “431” skin sample.

Histograms present cells treated with dead spores of *R. microsporus* (I), and cells treated with live spores of *R. microsporus* (II). There is noticeable maturation of dermal dendritic cells basing on CD83 expression after both, dead and live spore interaction with cells. This figure was analysed by Matt Ivory (Cardiff University).

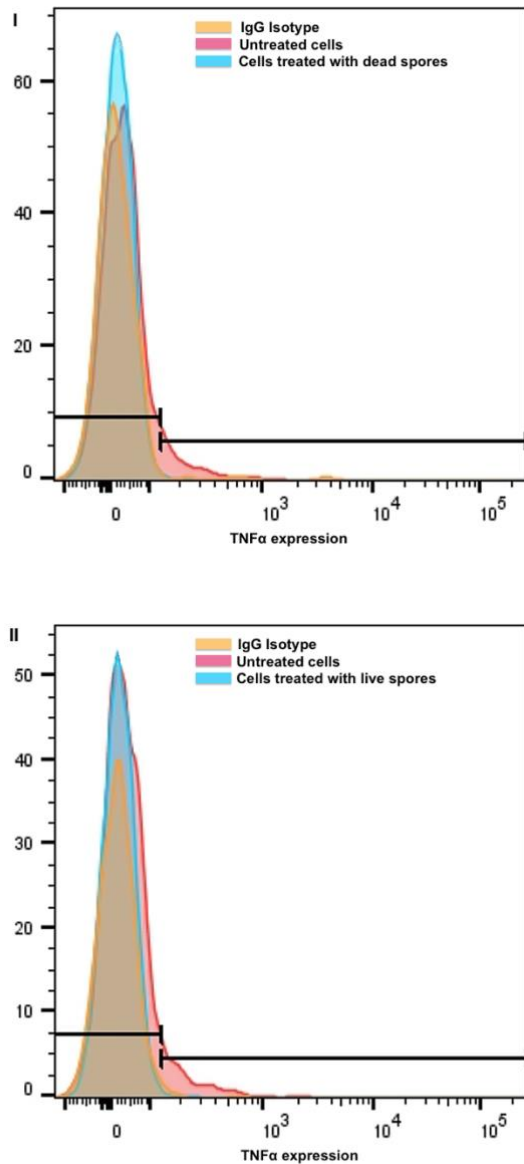


Figure 5.8 (b) TNFα expression of dermal cells from “431” skin sample.

Top histograms present cells treated with dead spores of *R. microsporus* (I), and cells treated with live spores of *R. microsporus* (II). There is no TNFα expression in cells treated with dead/live spores. This figure was analysed by Matt Ivory (Cardiff University).

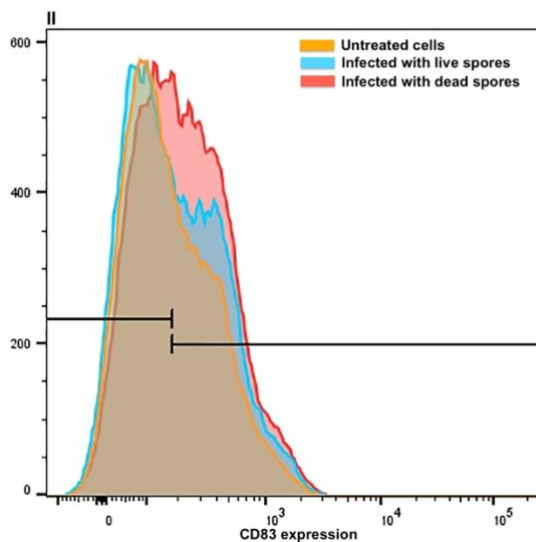
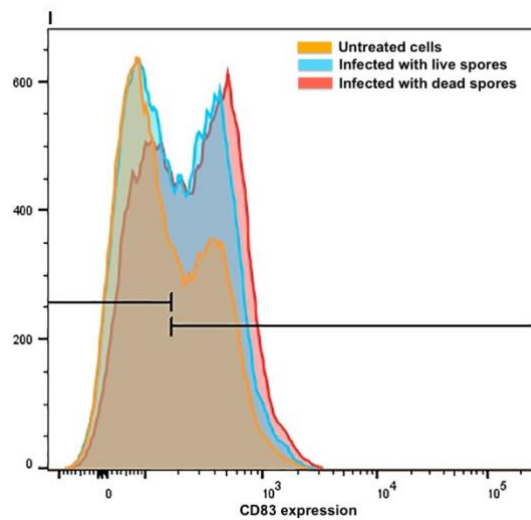


Figure 5.9 (a) CD83 expression of dermal cells from “434” (I) and “435” (II) skin samples.

There is recognizable maturation of dermal dendritic cells in both of the samples following infection with both, live and dead spores of *R. microsporus*. This figure was analysed by Matt Ivory (Cardiff University). Both, Wioleta Trzaska and Matt Ivory undertook this experimental procedure.

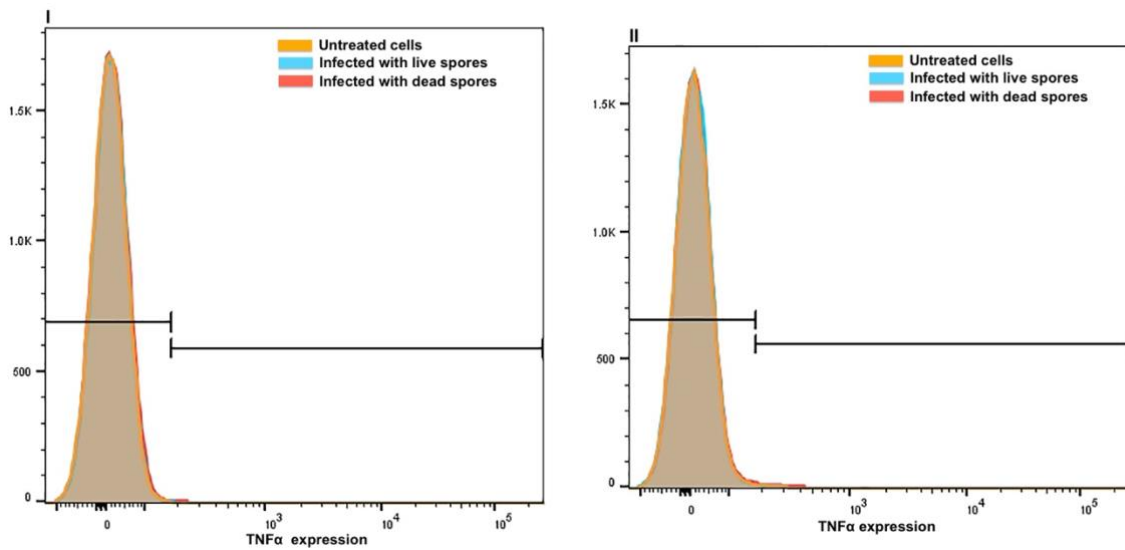


Figure 5.9 (b) TNFα expression of dermal cells from “434” (I) and “435” (II) skin samples.

There is no recognizable TNFα expression in dermal dendritic cells in both of the samples following infection with both, live and dead spores of *R. microsporus*. This figure was analysed by Matt Ivory (Cardiff University). Both, Wioleta Trzaska and Matt Ivory undertook this experimental procedure.

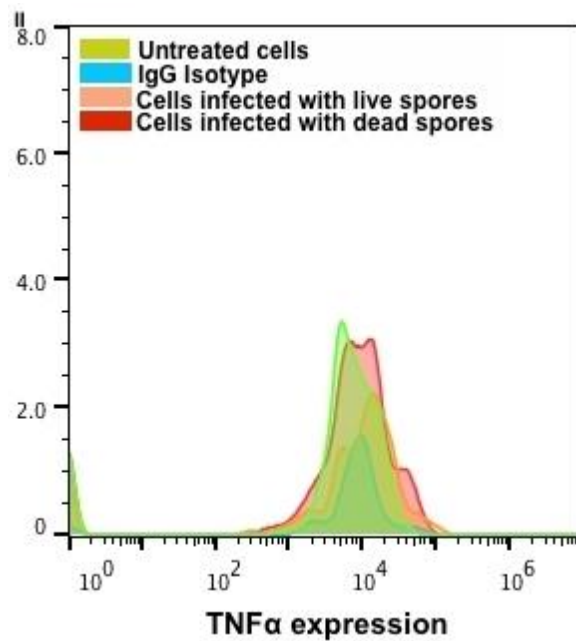
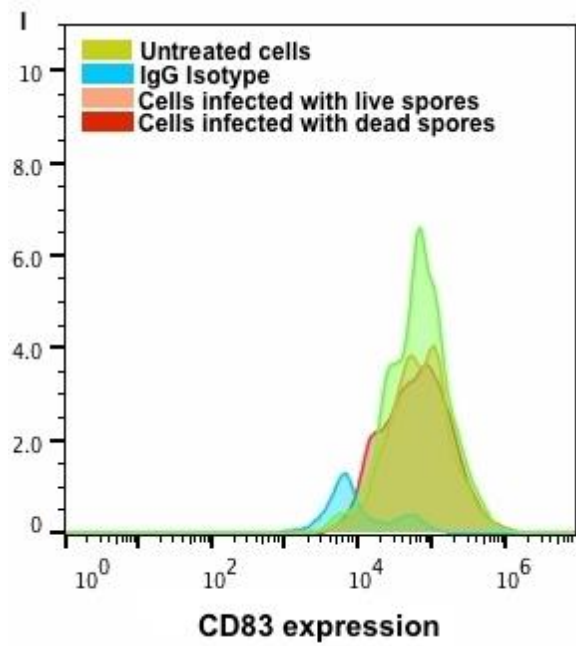


Figure 5.7 (b) CD83 and TNF α expression of dermal dendritic cells from “001” skin sample.

There is no dermal dendritic cells recognizable maturation CD83 (I) and no TNF α (II) expression following infection with spores of *S. apiospermum* 14462.

5.4 Discussion

The biggest organ of the human body, skin, is an excellent barrier to protect us from environmental pathogens as well as microbes resident on the skin. Apart from its mechanically protective role, it is equipped with a complex system of immune cells.

Dendritic cells residing in the epidermis, called Langerhans cells, are critical antigen-presenting cells that penetrate the epidermal layer, searching for pathogens and then present them to T cells. Going deeper into the skin, in the dermal layer, the immune system relies on dermal dendritic cells. Those, similar to LCs, undergo maturation after recognition of pathogen and migrate through the dermal lymphatic vessels to skin-draining lymph nodes to localize T cells. An important point to mention is that LCs may mediate tolerance whereas dDCs are thought of as more proinflammatory (Mutyambizi et al., 2009).

Here we present preliminary data on developing a novel model to investigate host-pathogen interaction using *ex-vivo* skin off cuts. This is a promising model for two reasons; first, to provide a source of isolated epidermal and dermal cells for *in vitro* study and second, to provide a model to observe skin penetration by pathogens. At this stage, the data presented here are very preliminary, since acquisition of human samples proved challenging. However, these preliminary data demonstrate feasibility and highlight some interesting (albeit n=1) observations, such as the specificity of dDC maturation in response to *R. microsporus*. Future development of this model will, we hope, offer useful insights into the role of skin immune function in protecting against trauma associated fungal infection. Pearton et al. (2010) have shown *ex-vivo* skin model as a valuable model to observe LCs morphological changes following ID (intradermal) injection of H1 VLP (influenza virus-like particle) vaccine. Their results in

ex-vivo skin model (human environment) confirmed previous observations in animal model. Similarly, research done by Ivory et al. (2015) and Schaeffer et al. (2015) also using *ex-vivo* skin model to investigate LCs response to dengue virus (DENV) have shown skin-resident immune cells increasing inflammatory response to DENV infection. Czubala et al. (2016) has also investigated LCs (isolated from epidermis), interaction with HIV-1 from the same model and showed ability of LCs to decrease post-entry activity of HIV-1.

6. Conclusions

6.1. Novel approaches to treat trauma-associated fungal infections

Traumatic injuries, caused by man-made accidents, environmental disasters or those acquired during combat, can result in serious infections leading to high morbidity and mortality. Fungal spores, the point of interest of this research, are typically introduced into a wound during injury, where the available nutrients, together with a moist and warm environment, provides the ideal ecosystem for their growth. All fungal species tested in this research, such as *Mucormycetes*, *Scedosporium*, *Lomentospora*, *Fusarium* and *Cryptococcus* are ubiquitous environmental fungi, with a wide-range of antifungal resistance profiles and global incidence. Diagnosis is very often delayed; the need for specimen collection and investigation requires time (in military field hospitals taking up to 10 days). These limitations often result in aggressive surgical debridement as a precautionary measure, resulting in disability and amputations. Moreover, the cost of treating invasive fungal infections is extremely high.

Therefore, here we have investigated how two novel treatments; acetic acid and blue light, are effective against fungal species causing wound infections.

Acetic acid.

We have demonstrated that low concentrations of acetic acid can inhibit spore germination of *Mucormycetes*, *Scedosporium*, *Lomentospora* and *Fusarium* spp. as well as preventing growth of *Candida* and *Cryptococcus* spp. Indicating that acetic acid can be used as a potential treatment; for example, as a wound topical agent or dressing at the time of the injury, such as in field hospitals, where appropriate diagnosis and treatments are very often delayed. We have also shown that low concentrations of

acetic acid inhibit further growth of germinated conidia, indicating that this drug can also be suitable for disease treatment even if infections have already established.

Moreover, this pattern was conserved across different fungal species tested, in comparison to other currently used antifungals, for example, commonly used hydrogen peroxide, which has been shown to be ineffective in reducing growth of different microbes (Drosou. A and Kirsner. R, 2003). Our findings are equivalent to the recent findings of Halstead et al. (2015), and others, who have shown antimicrobial properties of acetic acid to burn wound infection bacterial pathogens such as *P. aeruginosa*, *A. baumannii*, *E. coli*, *K. pneumoniae*, *E. cloacae*, *P. mirabilis* and MRSA (Entani et al., 1998; Ryssel al., 2009; Halstead et al., 2015). Therefore, acetic acid presents a powerful therapeutic agent, for both bacterial and fungal pathogens. Additionally, as shown by Fraise et al. (2013) it is a stable treatment that is not affected by evaporation nor by inactivation by cotton swabs, therefore is an ideal dressing drug for a wound infection.

Despite the long-time debate regarding the mechanism of action of acetic acid this remains unsolved. We have been able to light up the direction of further investigation into this subject by showing that the activity of acetic acid is not due to its low pH, but a combination of both hydrogen ions and accumulation of acetate within the fungal cell. Previous studies by Levine and Fellers (1940) have also shown that the acetic acid inhibitory effect is not due to a low pH, and also more recent research of Roe et al. (2002) who investigated acetic acid inhibitory effect of *E. coli*, have shown that this effect is a result of either reduction of pH or intracellular accumulation of acetate anion. Their research suggest that acetate inhibits the methionine biosynthesis pathway, which leads to the accumulation of homocysteine that has an inhibitory effect

to the cell (Roe et al., 2002). Also, Cottier et al. (2015 a, b) have shown transcriptional responses and *MIG1* gene involvement in the mechanism of action of weak acids as well as iron uptake involvement (Mira et al., 2010; Cottier et al., 2015 a, b). All this research suggests that the mechanism of action of acetic acid is multifactorial and it might depend on different factors such as, lowering intracellular pH, acetate accumulation within cell, gene up/down regulation, ion transport etc... (Hirshfield, Terzulli & O'Byrne., 2003).

Results obtained in this study highlight a great potential of acetic acid antifungal abilities to treat trauma-associated fungal infections, however this research holds a few limitations. First, the limited number of acids (lactic and hydrochloric acids) comparison to the activity of acetic acid itself, especially an organic acids such as citric, propionic, fumaric, sorbic or benzoic acids which are often of an interest of antimicrobial study (Hirshfield, Terzulli & O'Byrne., 2003). Second, a lack of investigation of potential acetic acid resistance that could be developed by the fungal species tested. It has been previously shown that *E. coli* and *Salmonella* have adapted to organic acids antimicrobial properties (Guilfoyle & Hirshfield., 1996; Kwon & Ricke., 1998). Therefore; a possibility of adaptation and/or resistance development to acetic acid should be investigated in fungal species used in this research. Third, we have shown that acetic acid is fungistatic and fungicidal against planktonic ungerminated and germinated cells/spores, but the activity of this acid has not been tested against fungal biofilms. It is well known that many fungal species including *Scedosporium/Lomentospora* spp., *Mucormycetes* spp. and *Fusarium* spp. can form biofilm (Mello et al., 2016; Singh, Shivaprakash & Chakrabarti., 2011; Peiqian et al.,

2014). Therefore; acetic acid activity should be tested on biofilms formed by those fungal species, its fungistatic/fungicidal concentration, stability and persistence.

In the future, it would be of benefit to explore in more depth the molecular mechanism of action of acetic acid. One of the approaches basing on our current results and observations (showing that acetic acid antifungal activity is a function of both undissociated acid and/or free acetate, together with the reduced pH driven by the dissociated hydrogen ions) would be to investigate fungal proton pump response to acetic acid. As this is an essential for intracellular pH regulation and maintenance of nutrient uptake, investigation of how acetic acid negatively affects its role could be an important point for understanding the antimicrobial assets of this acid. Two, main proton pumps involved in fungal pH control are V-ATPases and P-type proton pump Pma1 (Kane., 2016). Investigation of genetic regulation of those proton pumps during exposure to acetic acid could help identify target genes that are involved in up/down regulation of activity of proton pumps during such a treatment. This could help us develop novel drugs that would have a mechanism of action similar to powerful acetic acid that could be potentially used intravenously, not just topical as acetic acid is at the moment. Or/and identify how acetic acid could be designed to be used as oral/intravenous agent antifungal drug.

Blue light.

We have shown that blue light irradiation is effective against some but not all fungal species tested. These results are similar to those presented by Moorhead al. (2016), who investigated antifungal activity of blue light (405 nm) radiation on microcondia of *Trichophyton* spp. and *Aspergillus niger*. They showed that

microconidia of *T. rubrum* are much more sensitive to BL irradiation than the conidia of *A. niger*, so therefore species-specific activity of blue light (Moorhead et al., 2016).

We have also shown that blue light antifungal treatment was species-specific when treatment was applied on ungerminated spores/cells, but effective across all species when applied on germinated spores/cells. These results are similar to those presented by De Lucca et al. (2012) who have also shown that blue light treatment (20-100 J/ cm²) of ungerminated conidia of *Penicillium digitatum* is ineffective, however; they have noticed approximately 80-98 % viability losses when blue light treatment (40-100 J/ cm²) was applied on germinated conidia on this strain. A similar pattern was observed for *Fusarium graminearum* when blue light treatment was ineffective against ungerminated conidia, but effective on germinated ones (De Lucca et al., 2012). A difference between our studies was the amount of blue light delivered to fungal spores, we have treated the spores for 60 min which was equivalent to a dose of 216 J/ cm², while De Lucca et al. (2012) has applied blue light treatment with dose of 20-100 J/ cm². So, even with the higher dose provided by us caused no change in pattern of antifungal activity. Similar, species-specific result are found in bacteria and bacterial biofilms formation treatment of blue light. Halstead et al. (2016) have shown that Gram positive biofilms were less sensitive to blue light treatment than Gram negative biofilms. However, the authors conclude that despite variability of results between different bacterial isolates, overall results demonstrated blue light antimicrobial potential and decreased bacterial viability following treatment (Halstead et al., 2016).

Moreover, for the first time we showed that blue light induces morphological changes in *R. microspores*. It has been shown previously in one of the case study, that

Mucor circinelloides has been isolated from blood of immunosuppressed patient and was able to convert into yeast forms under anaerobic conditions (Arroyo et al., 2016). This phenomenon or ability of switching morphology is not understood so it might have immunological consequences that should be investigated further. As morphology has been shown in other fungi to have a major role in pathogenicity and virulence (Boyce & Andrianopoulos., 2015; Gauthier, 2017; Dambuza. et al., 2018). In our research, treating spores of different fungal species with blue light (BL), we have shown that this approach cannot be used as a prevention of a disease nor as a surface cleaner in the context of fungal pathogens. As blue light is already in use in many clinical settings, this research highlights both the advantages as well as potential risk of using such an approach.

Despite those interesting findings, we have faced a few pitfalls such as secondary heat generation and morphology switch in *Mucormycetes* spp. This study could be extended into a few additional experiments. One of those would be testing blue light activity against fungal species (*Mucormycetes*, *Fusarium*, *Scedosporium/Lomentospora* and *Candida* spp.) over time, to assess their susceptibility/resistance to regular treatment which could possibly be more effective than one dose treatment. This approach is being taken in terms of using blue light treatment for *Propionibacterium acne* causing facial acne. Gold et al. (2009) have presented study of treatment of mild-to-moderate inflammatory acne over the period of eight weeks on 21 volunteers. Their study showed that self-treatment using blue light device have reduced the number of inflammatory acne lesion significantly. This reduction was observed in time, more treatment, less blackheads and whiteheads appearance; skin irritation has also improved following treatment; sign of inflammation

have reduced in time too (Gold et al., 2009). Therefore, using a blue light device to treat trauma associated fungal species while they are still in spore/cell stage (ungerminated) would be interesting to investigate whether longer exposure/ more regular treatment to this light could have a more effective antifungal effect. Saying that, this approach would also be beneficial in terms of investigation of fungal adaptation/ resistance development. Long-term exposure would help us answer both questions; possible better antifungal efficiency but also, a possible fungal adaptation to this treatment. Another limitation of this study, a tactic to extend the research, would be cytotoxicity testing on epithelial/skin cells. There has been some research that present blue light not cause DNA damage nor early photo-ageing (Kleinpenning et al., 2010). Their research on healthy volunteers over time of 5 days have also shown that blue light irradiation caused transient melanogenesis and inexplicable vacuolization without resulting apoptosis (Kleinpenning et al., 2010). Similarly, research by Zhang et al. (2014) have shown that irradiation by blue light caused almost no apoptotic cells in epidermis. However, those researches have not been investigated on wound damaged skin, therefore this approach, using *ex-vivo* skin model together with blue light could bring a new set of data that would give us more knowledge of potential blue light treatment of trauma-associated fungal infections.

Future prospective for this project could involve an investigation of its molecular mechanism, which would be beneficial to answer why some fungal species are more susceptible to this treatment than others and potentially how this strategy may be improved. Our results suggest that one of the mechanisms involved in antifungal efficiency of BL could involve melanin presence/absence in fungal cell wall. It is well known that melanin plays an important role in fungus protection from extreme

environmental conditions but also is involved in virulence (Gessler, Egorova & Belozerskaia., 2014). *A. fumigatus*, for example, uses melanin to inhibit phagolysosome acidification and to protect itself from UV (Geib et al., 2016). *L. prolificans* it has been shown to use melanin to protect itself from UV, H₂O₂ but not ampB (Al-Laaiby et al., 2016). It is also known that cell wall of many fungal species changes during germination, for example *Mucor* of *Mucormycetes* spp. melanin content is different between conidial cell wall and hyphae, for example (Orlowski, 1991). That could be an explanation for different susceptibility of those species to blue light treatment. But this requires further investigation.

6.2 Investigation of host-pathogen interactions

Our understanding of how fungal pathogens interact with host innate immune cells and how they manipulate those cells is still very limited, despite many years of research. In particular, some of the species associated with traumatic injuries, such as *Scedosporium* and *Lomentospora* spp., are very understudied.

***Scedosporium* and *Lomentospora* spp. interaction with the host innate immune system.**

Here we have investigated how highly pathogenic and antifungal resistant species of *Scedosporium* and *Lomentospora* interact with innate immune cells. We have shown that both *S. apiospermum* and *L. prolificans* are well phagocytosed by a range of different macrophages; including the murine macrophage cell line J774A.1, primary cells such as HMDMs, and iPSC- derived macrophages. These results are similar to Xisto et al. (2016) who have also shown, that *L. prolificans* is being well phagocytosed by murine macrophages. They have shown that O-linked

oligosaccharides are involved in recognition, uptake and pro-inflammatory cytokine production by macrophages when in contact with *L. prolificans* (Xisto et al., 2016). Gil-Lamaignere et al. (2003) have also shown that *S. apiospermum* is being well phagocytosed by monocyte-derived macrophages. They have presented in their research, in between, that serum opsonisation of hyphae had higher level of superoxide anion (O_2^-) release by human polymorphonuclear leukocytes (Gil-Lamaignere et al., 2003). We have, in fact, not investigated how serum opsonisation influence hyphal damage to compare to their results, but we have research whether serum opsonisation will influence fungal spore survival within macrophages, and we found no difference in survival when compared to control (non-opsonised). The answer to why serum opsonisation had no influence of fungal survival remain unknown, however we could assume/propose further investigation on complement receptors that are potentially not involved in phagocytic uptake or/and fungal survival within macrophages, and signalling pathways involved in this process. In all cases, however, the fungi are able to survive and then subsequently germinate, penetrate and kill the macrophage. We have also shown that live spores of *S. apiospermum* and *L. prolificans* manipulate J774A.1 by preventing acidification of the macrophage phagosome. The question of what approach those fungal species take to do so remain unanswered at the moment by us as well as other research groups. One striking finding is that *L. prolificans* is consistently better at germinating within macrophages than *S. apiospermum*. These results were no surprise to us as it has been previously shown that *L. prolificans* have higher germination rate generally, and within innate immune cells than *Scedosporium* spp. (Mello et al., 2018; Rollin-Pinheiro et al., 2017). Moreover, for the first time, we have shown that vomocytosis and lateral transfer are

two distinct events by the observation that *Scedosporium* and *Lomentospora* spp. appear unable to vomocytose and yet still undergo lateral transfer.

During investigation of *Scedosporium* and *Lomentospora* spp. interaction with macrophages we met a few pitfalls. The main one was a lack of time to investigate some of the matters in detail. These are signalling pathways involved in spore survival within macrophages that could have been involved in either: serum opsonisation results that we have gained, or ability of spore to manipulate acidification by phagosome to enable fungal survival. We have also only touched on the investigation of events such as vomocytosis and lateral transfer in both, *Scedosporium* and *Lomentospora* spp. All of those mentioned above are complex behaviours that requires many experimental approaches to be able to seek an answer to potential explanation and requires to be investigated further.

Based on this research, one of the most interesting results worth further investigation is the mechanism of action behind lateral transfer and vomocytosis. However, we now know that some fungi are able to escape from the phagocyte and evade killing by two processes, non-lytic expulsion (vomocytosis) and cell-cell transfer (lateral transfer). Despite the discovery of these processes over 10 years ago (Ma et al., 2006; Ma et al., 2007; Al-Laeiby et al., 2016), the driver(s) behind and reason(s) for the existence of these mechanisms of escape are largely unknown. In the past, vomocytosis has been described as unsuccessful lateral transfer where the phagocyte fails to pass the fungus to another phagocytic cell (Ma et al., 2006). Because *Scedosporium* and *Lomentospora* spp. are first to be known fungal pathogens that are able to perform one (lateral transfer) but not the other, are a great model to investigate those two events as a separate incidents. This preliminary data shows macrophages

infected with *Scedosporium* and *Lomentospora* or *Cryptococcus* spp. act cell to cell differently. There were no vomocytosis events when macrophages are infected with either *Scedosporium* or *Lomentospora* spp. However, there were recurring events of lateral transfer. More interestingly, spore transferred to another macrophage was either killed in the recipient macrophage or germinated. Moreover, macrophages that transfer a spore to another macrophage was not necessarily necrotic.

Future research would also address how these uncharacterized fungi interact with the innate immune system, and provide answers regarding the driving force behind lateral transfer and vomocytosis. This increased knowledge could be exploited in subsequent applications to develop novel approaches to tackling fungal infections.

Ex-vivo skin model.

The second part of this host-pathogen investigation in this project was to develop a novel ex-vivo skin model. As breached skin is the first organ that comes into contact with environmental pathogens at wound sites, an investigation of how skin immune cells interact with fungi is important for determining the host response to traumatic infection. Both skin layers, epidermis and dermis, are equipped with antigen presenting cells who undergo maturation after pathogen recognition, and present pathogen antigen to T cells. However, it remains unknown how the cells in these two layers respond to *R. microsporus* and *S. apiospermum*.

Therefore, here we investigated if both Langerhans cells (cells of the epidermis) and dermal dendritic cells (dermal cells) are able to recognise fungal pathogens, undergo maturation and release cytokines. We have chosen to investigate CD83 marker, a maturation marker of dendritic cells, and TNF α , a cytokine released by DCs

to stimulate T cells. Our results are very limited due to the very early stage of this study. However, it appears that LCs do not mature or release TNF α following infection with *R. microsporus* and *S. apiospermum*. One explanation for this finding is that LCs are found in the top layer of the skin and thus might contact environmental pathogens on a regular basis and therefore should show tolerance towards them. These results may be equivalent to other researcher's investigation on LCs tolerogenic role (Mutiyambizi, Berger & Edelson., 2009; Shklovskaya et al., 2011; Kitashima et al., 2018). However, dDC do recognise *R. microsporus*, but not *S. apiospermum* (n of 1), and undergo maturation but interestingly do not appear to release detectable TNF α , a finding which, if reproducible, would hold significant clinical implications. *Ex-vivo* skin model, however recently appear more often in research, especially in investigation of viral infections/entry to our body (Schaeffer et al., 2015; Czubala et al., 2016) is still not well understood and very complex. More research on skin cells subsets as well as its interaction with a variety of different pathogens, such as fungal species whose are being in contact with traumatic wound, will help us to gain more knowledge about those cells that are very often first contact with environmental pathogens on a daily basis.

As mentioned above, this model is at a very early stage of study. During this study we met an issue with obtaining skin samples. Due to this, we cannot base any conclusions gained from our results, however we can propose this model as a valuable *ex-vivo* model to investigate host-pathogen interaction.

Future investigation, if more skin samples was be provided, would be to take dual approaches. First, to continue investigate LCs and dDCs, isolated from skin sheets, interaction with fungal pathogens, with extension to explore different cytokines that are potentially released from immune cells in contact with fungal pathogens, as

well as investigate more trauma associated fungal species. Second approach would be to use *ex-vivo* skin off cuts as a model to investigate how fungal pathogens penetrate skin using biopsy methods. This method would allow as seeing how immune cells gathering (or not) around the pathogen and migrating down to present antigens to T cells. Moreover, this approach would help us to answer some question and link to previous research questions/topics we raised in this PhD project. Those would be to use skin sheet samples to infect with fungal spores and investigate how acetic acid can be used as a topical treatment/wound dressing and to compare its antifungal activity to *in-vitro* results that we have obtained. We would extend the methodology into using histopathology to observe changes in skin cells following contact with fungal pathogens, similar approach to that taken by Pearton et al. (2010). This observation would give us an idea of antifungal activity of acetic acid, penetration of drug through the wounded vs none wounded skin and the cytotoxicity on intact and wounded skin. Then, we would investigate the antifungal potential of blue light. Similarly, to acetic acid proposed approach, we would investigate if and how deep blue light penetrate skin samples infected with fungal spores. Its toxicity on wounded vs unwounded skin samples following multi-time treatment and possible combination of low concentration of acetic acid and BL treatment.

7. THESIS SUMMARY

This Thesis has been focused on investigation of novel treatments for fungal infections associated with traumatic injuries and understanding the host interaction with one of those, very understudied fungal species, *Scedosporium* and *Lomentospora*. The data in this thesis highlights great antifungal activity of acetic acid against different fungal species tested. The blue light approach is associated with more challenges, because my data suggest that it can have both positive and negative impacts on patient outcome, depending on the infecting species. This requires proper diagnosis prior to treatment. This research also shed light on lateral transfer and phagocytosis events to be separate mechanisms, shown here for the first time, during investigation of *Scedosporium* and *Lomentospora* spp. with macrophages. Finally, this research also introduce into *ex-vivo* skin model as a potential model to investigate skin-fungal interaction.

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9. PUBLICATIONS



pH Manipulation as a Novel Strategy for Treating Mucormycosis

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Mucormycosis is a fatal fungal disease caused by several organisms within the order Mucorales. In recent years, traumatic injury has emerged as a novel risk factor for mucormycosis. Current antifungal therapy is ineffective, expensive, and typically requires extensive surgical debridement. There is thus a pressing need for safe prophylactic treatment that can be rapidly and easily applied to high-risk patients, such as those with major trauma injuries. Acetic acid has been used as a topical treatment for burn wounds for centuries and has proven activity against Gram-negative bacteria. Here, we demonstrate that acetic acid is also highly effective against major pathogenic groups of Mucorales, even at very low concentrations (0.3%). This antifungal effect is not seen with other acids, such as hydrochloric and lactic acid, suggesting that acetic acid activity against Mucorales spores is not solely evoked by low environmental pH. In agreement with this, we demonstrate that the antifungal activity of acetic acid arises from a combination of its ability to potentially lower intracellular pH and from pH-independent toxicity. Thus, dilute acetic acid may offer a low-cost, safe, prophylactic treatment for patients at risk of invasive mucormycosis following traumatic injury.

Mucormycosis is an infection caused by fungi that belong to the order Mucorales, pervasive environmental fungi found in soil and decaying wood. Within the class Mucormycetes, the order Mucorales contains the genera *Rhizopus*, *Mucor*, *Rhizomucor*, and *Lichtheimia*, which cause most cases of human infection (1). Over the past two decades, the frequency of patients with mucormycosis has increased significantly (2). This increase in infections is associated with excessive morbidity and mortality, and it is directly related to an increasing patient population with underlying immunocompromising conditions which put them at risk for the development of serious fungal infections like mucormycosis (3). These include individuals suffering from uncontrolled diabetes mellitus, patients who have received an organ or hematopoietic stem cell transplant, patients with malignancy or neutropenia, and those who receive deferoxamine therapy or intravenous drug users (4, 5). Frequently, however, cases of mucormycosis also occur in immunocompetent patients who have received traumatic injuries in settings such as agricultural accidents, motor vehicle collisions, and blunt crush injuries, as well as during natural disasters (6). In addition, several cases have been reported recently in military personnel, secondary to blast injuries caused by improvised explosive devices (7).

Established mucormycosis has an extremely high mortality rate, in excess of 90% (4). Effective treatment of this disease thus depends on an early diagnosis and prompt initiation of therapy. Frontline antifungals used for treatment of mucormycosis include amphotericin B in various liquid formulations and posaconazole (8, 9). Amphotericin B is the more active agent but carries significant risks of organ toxicity. Posaconazole appears to be well tolerated and is recommended to be used for treatment in patients with amphotericin B intolerance or those unresponsive to previous antifungal therapy (10, 11). In high-risk patient groups (such as severely injured military personnel) prophylactic administration of posaconazole prior to diagnosis may be effective, but this is economically unfeasible in most settings. Thus, a cheap, safe, and easy-to-apply prophylactic treatment to reduce the incidence of mucormycosis in open wounds is likely to have significant clinical benefits.

Acetic acid has been used for thousand of years as an antimi-

crobial agent, for instance, to reduce the spread of plague in medieval times or as a wound dressing in the American Civil War (12). More recently, it has been used to treat burn wound infections, and it has demonstrable activity against several bacterial pathogens (13). To date, however, its activity against fungi in general and mucormycosis in particular has not been tested.

Here, we demonstrate that acetic acid has potent antifungal efficacy at very low concentrations against all Mucorales species tested. This activity is not seen for other acids. Using ratiometric fluorescence reporters, we show that fungal spores treated with acetic acid undergo rapid acidification of their cytoplasm and become arrested without germination.

Thus, we propose that early application of dilute acetic acid may represent an effective and low-cost strategy to minimize mucormycosis in traumatic wounds.

MATERIALS AND METHODS

Mucorales strains. Four different isolates were tested; *Lichtheimia corymbifera* 9.6002134 and *Rhizopus microsporus* 12.6652333, which are clinical isolates, and *Mucor circinelloides* NRRL3631 and *Mucor circinelloides* CBS277.49 (4). All strains were grown on Sabouraud agar plates for at least 10 days before use.

Media. Acetic acid experiments were performed in Sabouraud broth (Fisher Scientific) and complete RPMI medium 1640 (cRPMI), phenol free (Gibco), plus 100 U/ml of penicillin and 100 U/ml of streptomycin (Sigma), 2 mM L-glutamine (Sigma), and 10% fetal bovine serum (FBS)

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TABLE 1 pHs for different concentrations of acetic acid in Sabouraud medium

% acetic acid	pH
5	2.52
2.5	3.27
1.25	3.63
0.63	3.92
0.31	4.18
0.16	4.43
0.08	4.68
Sabouraud medium only (control)	5.73

TABLE 2 pHs for different concentrations of acetic acid in cRPMI medium

% acetic acid	pH
5	2.32
2.5	3.19
1.25	3.67
0.63	4.03
0.31	4.46
0.16	5.24
0.08	6.32
RPMI medium only (control)	7.45

(Sigma) with different concentrations of acetic acid (Tables 1 and 2). For hydrochloric and lactic acid treatments, the pHs in Sabouraud broth and cRPMI were matched with the pHs generated by the different concentrations of acetic acid. Spores were washed with phosphate-buffered saline (PBS), spun down, and resuspended in PBS for cell counting in a hemocytometer. Spore counts were adjusted to a final count of 1×10^4 cells per ml.

Fungal growth inhibition assays. Five percent acetic acid (Tayside Pharmaceuticals, Dundee, United Kingdom) was serially diluted into Sabouraud medium and cRPMI to produce concentrations of 2.5%, 1.25%, 0.63%, 0.31%, 0.16% and 0.08%. The pH of each of these concentrations of acetic acid was measured, and then pH-matched solutions of hydrochloric acid (Fluka) or lactic acid (Sigma) were produced in Sabouraud and cRPMI media (Tables 1 and 2). For pH-neutralized experiments, media were generated as described above, but then, 1 M NaHCO_3 was added to return the pH to that of the control. Fungal growth rate was measured by reading the optical density at 600 nm (OD_{600}) at time zero and after 24 and 48 h using a microplate reader (BMG Labtech). Plates were incubated at 37°C in between reads, without shaking. To estimate spore killing, samples from cultures with the concentrations of acetic acid in which no growth was observed were plated onto Sabouraud agar plates, and the numbers of CFU were counted 24 h later. Each experiment was performed with at least three experimental replicates and three biological repeats.

Time-lapse imaging. Time-lapse imaging was performed on fungal spores either prior to or shortly after germination, using a Nikon Eclipse Ti microscope with a long working distance (LWD) 0.5220× objective for 18 h. Movies were analyzed and prepared for publication using NIS Elements software.

Time of action. To test how rapidly acetic acid affects fungal viability, spores were incubated for 0, 15, 30, 60, 120, 180, 240, and 300 min in 2.5% acetic acid (in cRPMI) and then plated onto Sabouraud plates for subsequent colony counting 24 h later. Scoring for CFU at this early time point requires more careful observation but avoids the problem of extensive filamentation that leads to fused colonies.

Measurement of pH_i. Fungal spores at a concentration of 6×10^6 /ml were incubated in RPMI medium at 37°C and 5% CO_2 for 4 h before imaging. Cells were then incubated with 5 μM BCECF-AM [2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester; Life Technologies, USA] for 1 h and resuspended in fresh medium to allow full deesterification of the dye. Intracellular pH was measured via single-cell ratiometric imaging at $\times 60$ magnification using an Olympus IX81 (Olympus, United Kingdom) coupled to a monochromator-based illumination system (Cairn Research, United Kingdom) and an Evolve 512 EMCCD (Photometrics, USA) digital camera; image acquisition was controlled using MetaFluor (Molecular devices, USA) acquisition software. Fluorescence emission for excitation wavelengths centered at 490 nm and 436 nm was captured at 530 nm, and ratios were obtained for individual cells after background subtraction. Ratios were converted to intracellular pH values after *in situ* calibration as described by James-Krakke (14). Briefly, after the initial germination stage, cells were permeabilized in

RPMI supplemented with 100 μM nigericin and 150 mM KCl and subsequently exposed to extracellular pHs ranging from 4.5 to 7.5. BCECF-AM ratios were then converted to intracellular pH (pH_i) values using the following equation: $\text{pH}_i = \text{pK}_a - \log[(R_{\text{max}} - R)/(R - R_{\text{min}})] \times F_{\text{base436}}/F_{\text{acid436}}$, where pK_a is the acid dissociation constant for BCECF-AM, R is the ratio of the emission fluorescence signals measured at 530 nm when the fluorophore is excited at 490 nm and 436 nm, respectively, R_{max} is the ratio for maximum fluorescence measured at pH 7.5, R_{min} is the ratio for minimum fluorescence measured at pH 4.5, and $F_{\text{base436}}/F_{\text{acid436}}$ is the ratio of fluorescence signals at 436 nm under the basic and acid conditions used to obtain R_{max} and R_{min} .

RESULTS

Acetic acid shows strong antifungal activity against Mucorales species. Ubiquitous fungal spores are the infectious agent for mucormycosis. Symptomatic mucormycosis depends on germination of fungal spores and subsequent tissue-invasive hyphal growth. To test whether spore germination was inhibited by acetic acid, spores of *Rhizopus microsporus* 12.6652333, *Lichtheimia corymbifera* 9.6002134, and *Mucor circinelloides* NRRL3631 and CBS277.49 (4) were inoculated into Sabouraud broth (to mimic nutrient-rich conditions) and cRPMI (containing serum to test for potentially inhibitory effects) with various acetic acid concentrations. Mucorales grow as filamentous fungi and, thus, OD measurement is problematic for monitoring growth rates but is very successful in monitoring the onset of germination. Acetic acid impaired germination in all isolates at concentrations as low as 0.3% (Fig. 1 and 2; see also Fig. S1 and Movies S1 to S5 in the supplemental material).

To test whether this effect was fungistatic or fungicidal, spores were plated onto Sabouraud agar plates and the number of colonies was counted after 24 h. At concentrations of 2.5% and above, no viable colonies could be recovered (Fig. 3; see also Fig. S2 in the supplemental material). Time course analysis indicated that this fungicidal activity peaks following approximately 4 h of exposure to 2.5% acetic acid (Fig. 4). Thus, acetic acid strongly suppresses fungal germination at very low concentrations and is potentially fungicidal at concentrations above 2.5%.

Antifungal activity against Mucorales is not seen with other acids. To investigate whether this effect is driven solely by environmental pH, we performed similar experiments using an alternative organic acid (lactic acid) and an inorganic acid (hydrochloric acid). We matched the pHs of Sabouraud medium seen at different concentrations of acetic acid with both hydrochloric and lactic acid (Tables 1 and 2) and monitored the cultures for spore germination. Neither hydrochloric nor lactic acid (Fig. 5; see also Fig. S3 to S5 in the supplemental material) significantly inhibited spore germination other than at the very lowest pHs. Thus, the

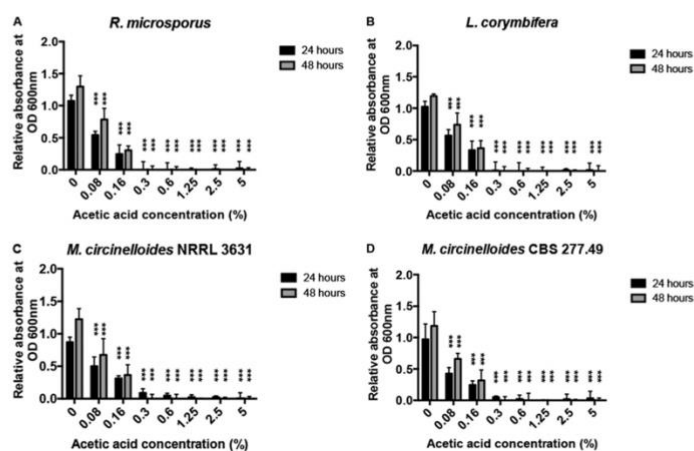


FIG 1 Acetic acid inhibits fungal spore germination. Spores were grown in Sabouraud medium, and growth was assessed by OD₆₀₀ measurements. Graphs show OD measurements after 24 and 48 h. Error bars represent standard deviations ($n = 4$, with three experimental replicates at each time point); statistical analysis was conducted using two-way analysis of variance (ANOVA) with Dunnett posttest. ***, $P < 0.001$.

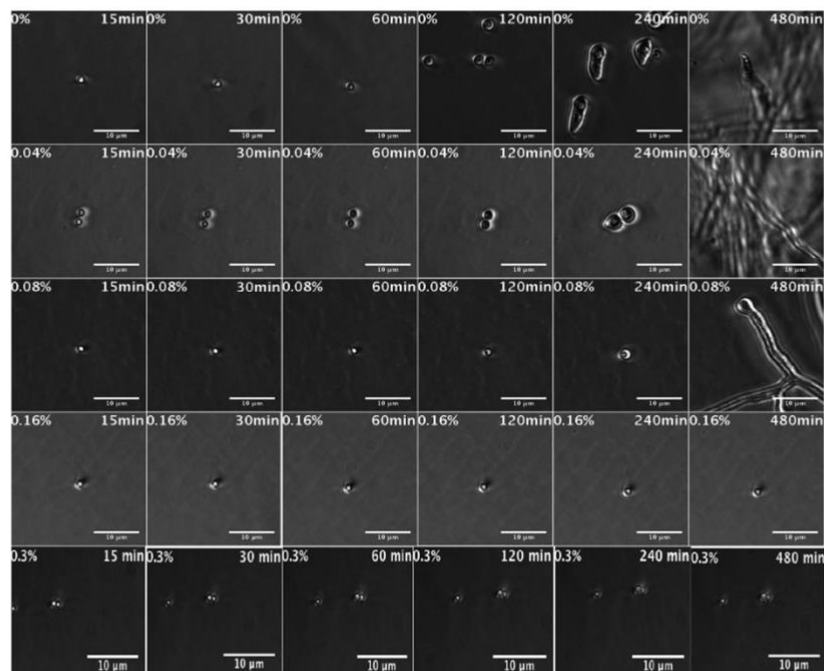


FIG 2 Visualization of acetic acid activity against *R. microsporus*. Time-lapse imaging shows that acetic acid concentrations of 0.16% or above strongly inhibit germination. Frames are extracted from Movies S1 to S5 in the supplemental material.

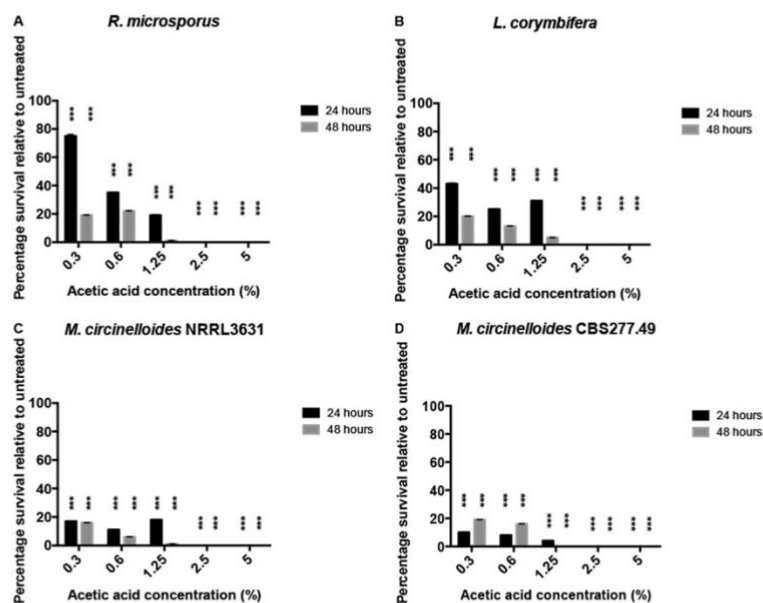


FIG 3 Acetic acid is fungicidal to spores at concentrations of 2.5% and above. Spores were grown in Sabouraud medium and then plated after 24 h of incubation for CFU counts. Graphs show CFU/ml. Error bars represent standard deviations ($n = 3$, with three experimental replicates at each time point); statistical analysis was conducted using two-way ANOVA with Dunnett posttest. ***, $P < 0.001$.

antifungal effect of acetic acid is not simply a reflection of lowered environmental pH.

Acetic acid inhibition of spore germination is not solely due to reduced intracellular pH. To investigate the mechanism by which acetic acid inhibits spore germination, we measured the

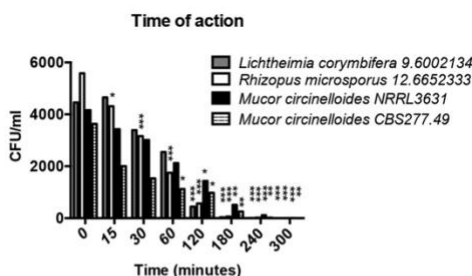


FIG 4 The fungicidal activity of 2.5% acetic acid requires prolonged incubation. Spores were grown in RPMI medium supplemented with 2.5% acetic acid and plated onto Sabouraud agar plates every 15, 30, 60, 120, 180, 240, and 300 min. Numbers of colonies were counted 24 h later. Error bars represent standard deviations ($n = 3$, with three experimental replicates at each time point); statistical analysis was conducted using two-way ANOVA with Dunnett posttest. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

intracellular pH of fungal spores during exposure to acetic, hydrochloric, and lactic acid. Cells were incubated with the pH-responsive BCECF-AM dye and monitored following acid exposure. At identical extracellular pHs, acetic acid lowered intracellular pH more strongly than hydrochloric or lactic acid, likely due to stronger dissociation of the ions within the fungal cytoplasm (Fig. 6; see also Fig. S6 in the supplemental material). However, even at identical intracellular pHs, acetic acid is far more effective at inhibiting fungal germination, suggesting that this inhibition is not solely due to the raised intracellular hydrogen ion concentration (e.g., compare growth inhibition by 0.3% acetic acid in Fig. 1 with growth inhibition by hydrochloric acid at pH 3.56 in Fig. 5, both of which drive an intracellular spore pH of 5.9, as shown by the results in Fig. 6).

Growth inhibition by acetic acid involves both pH- and acetate-dependent effects. To test a potential pH-independent effect of acetic acid, we neutralized different concentrations of acetic acid by adding NaHCO_3 to return the pH to that of the control but retain the presence of free acetate. These pH-neutralized media continued to inhibit spore germination (Fig. 7), less effectively than nonneutralized acetic acid but more effectively than other acids. Thus, the suppression of fungal germination by acetic acid involves both pH-dependent and pH-independent but acetate-dependent mechanisms.

Acetic acid shows activity against actively growing Mucorales. In a clinical setting, Mucorales spores may have germinated

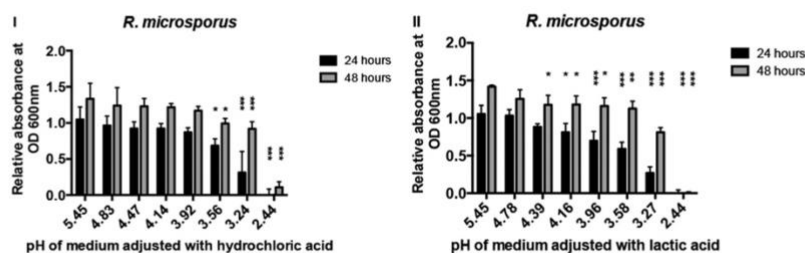


FIG 5 Other acids are less effective at inhibiting spore germination. Neither hydrochloric (I) nor lactic (II) acid was able to inhibit spore germination as effectively as acetic acid. Spores were grown in Sabouraud medium, and growth was assessed by OD₆₀₀ measurements. Graphs show OD measurements after 24 and 48 h. Error bars represent standard deviations ($n = 3$, with three experimental replicates at each time point); statistical analysis was conducted using two-way ANOVA with Dunnett posttest. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

before treatment can be initiated. Thus, to investigate whether acetic acid is active against germinated spores, we monitored filamentously growing fungal cells by time-lapse microscopy and then exposed them to different concentrations of acetic acid (Fig. 8; see also Movies S6 to S9 in the supplemental material). As with nongerminated spores, growing fungi were strongly inhibited by acetic acid concentrations as low as 0.3% (Fig. 8). Thus, acetic acid is effective even against spores that have already germinated.

DISCUSSION

Here, we describe the fungistatic and fungicidal properties of acetic acid against several mucormycete species.

Mucormycosis is a life-threatening infection, with mortality rates of more than 90%. While susceptible patient groups (e.g., those with impaired immunity and traumatic injuries) are increasing, diagnosis is often delayed and antifungal therapy typically ineffective and expensive. Hence, there is a need for prompt prophylactic treatment for high-risk patients (e.g., severely injured patients). Therefore, we have investigated the efficacy of acetic acid, a traditional method used in medicine, as a topical treatment for this fatal fungal infection.

We here demonstrate that concentrations of acetic acid as low as 0.3% are able to inhibit the spore germination and fungal growth of four isolates, representing diverse Mucorales species (*Rhizopus microsporus* 12.6652333, *Lichtheimia corymbifera*

9.6002134, and two isolates of *Mucor circinelloides*, NRRL3631 and CBS277.49) (4).

Very early studies of acetic acid activity against bacteria by Levine and Fellers (15) suggest that acetic acid toxicity is not due to hydrogen ion concentration alone but seems to be a function of the concentration of undissociated acid. Such a model is supported by our observation that acetic acid is more potent antifungal than other organic and inorganic acids (lactic and hydrochloric acid, respectively), even when matched for extracellular or intracellular pH. Thus, the antifungal activity of acetic acid seems to be a function of both hydrogen ion concentration and undissociated acid, or free acetate. As undissociated acid molecules diffuse through the cell membrane, they dissociate further within the cytoplasm. Thus, the fungus must expend energy both to pump out excess protons and to deal with free acetate within the cytoplasm.

Acetic acid is an abundant and cheap compound that is temperature stable and nonhazardous. It is therefore extremely amenable to use as a topical antifungal in challenging clinical situations, for instance, as a topical wound dressing for military personnel or those working in remote areas where Mucormycete infection is likely. Since it remains highly effective even at low concentrations, there is very limited risk of adverse effects or patient discomfort. Thus, our data suggest that clinical trials to test the efficacy of rapid, topical application of acetic acid are war-

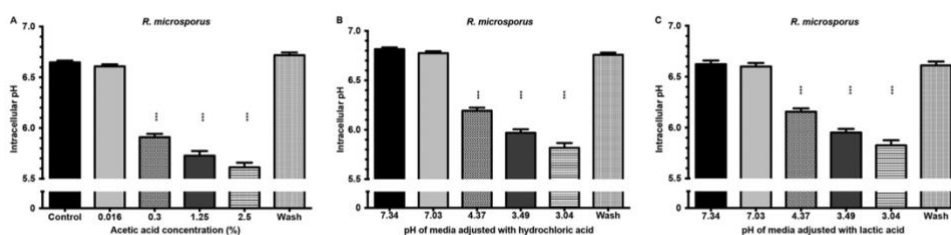


FIG 6 Intracellular pH measurements following treatment with different acids and, following experimentation, upon return to neutral pH via a wash with buffered medium. Spores were grown in RPMI medium supplemented with different acids. The intracellular pH was measured using the calibrated ratiometric analysis of the BCECF-AM dye described in Materials and Methods. Error bars represent standard errors of the means ($n = 3$, with three experimental replicates at each time point); statistical analysis was conducted using two-way ANOVA with Dunnett posttest. ***, $P < 0.001$.

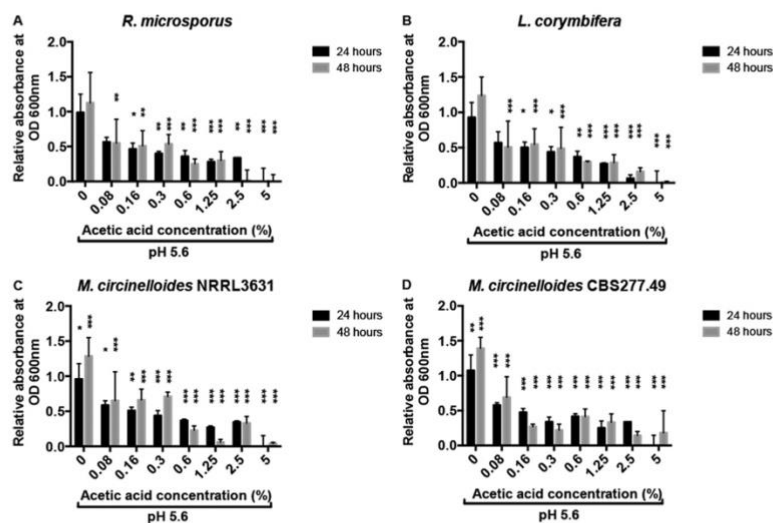


FIG 7 Neutralized acetic acid inhibits fungal spore germination with moderate efficacy. Spores were grown in Sabouraud medium, and growth was assessed by OD₆₀₀ measurements. Graphs show OD measurements after 24 and 48 h. Error bars represent standard deviations ($n = 4$, with three experimental replicates at each time point); statistical analysis was conducted using two-way ANOVA with Dunnett posttest. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

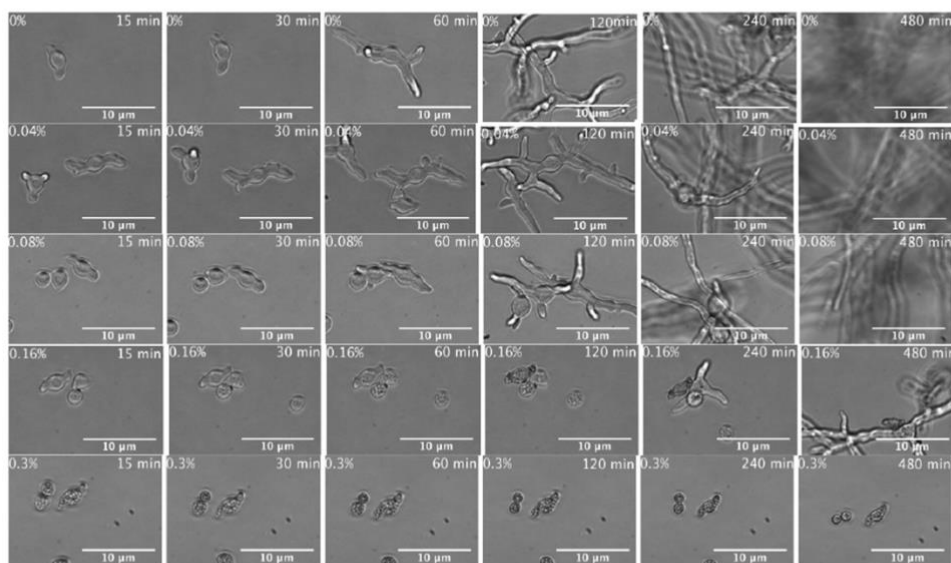


FIG 8 Acetic acid exerts an antifungal activity on pregerminated spores. Time-lapse imaging shows prolonged inhibition of further fungal growth in germinated spores at acetic acid concentrations of 0.3% or above. Time-lapse frames are derived from Movies S6 to S9 in the supplemental material.

ranted and that this treatment may offer an effective, low-cost prophylactic treatment for an infectious disease that is currently very challenging to treat.

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Species-specific antifungal activity of blue light

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Fungal pathogens represent a significant threat to immunocompromised patients or individuals with traumatic injury. Strategies to efficiently remove fungal spores from hospital surfaces and, ideally, patient skin thus offer the prospect of dramatically reducing infections in at-risk patients. Photodynamic inactivation of microbial cells using light holds considerable potential as a non-invasive, minimally destructive disinfection strategy. Recent data indicate that high-intensity blue light effectively removes bacteria from surfaces, but its efficacy against fungi has not been fully tested. Here we test a wide range of fungi that are pathogenic to humans and demonstrate that blue light is effective against some, but not all, fungal species. We additionally note that secondary heating effects are a previously unrecognized confounding factor in establishing the antimicrobial activity of blue light. Thus blue light holds promise for the sterilization of clinical surfaces, but requires further optimization prior to widespread use.

Invasive fungal infection is a common secondary complication of traumatic injury and can involve a wide-range of fungal species from diverse genera such as *Candida*, *Fusarium*, *Rhizopus* and *Scedosporium*, amongst others^{1–3}. Fungal spores present in the environment are easily introduced into wounds after traumatic injury such as motor vehicle accidents, environmental disasters or injuries resulting from military operations¹. Once established in the host, fungal infections are difficult to treat and are associated with high levels of morbidity and mortality. Thus strategies to decolonize hospital surfaces and surface-exposed wound tissue hold considerable promise for reducing secondary fungal infection.

Exposing microbes to a range of different light wavelengths, in combination with photosensitizing dyes, can effectively inactivate various bacteria, mycoplasma and viruses⁴. Such a combination is called photodynamic therapy (PTD) and has been clinically approved. However, a major challenge for PTD is the need to introduce exogenous photosensitizers into the pathogen⁵. More recently, however, there has been considerable interest in exploiting blue light, which appears to be effective against pathogens without the need for exogenous photosensitizers. In particular, Zhang and colleagues demonstrated the efficacy of blue light against several pathogens, including the intrinsically antimicrobial-resistant species *Acinetobacter baumannii*, in a mouse burn model of infection^{5,6}. Importantly, they also demonstrated that bacteria are more susceptible to blue light than keratinocytes, offering advantages over other currently used topical treatments that are often toxic and ineffective. The proposed mechanism behind the action of blue light is the photoexcitation of endogenous porphyrins, resulting in the production of ROS (Reactive Oxygen Species) and cell death⁷, although this has yet to be formally demonstrated for most blue-light susceptible organisms.

To date, very few fungal species have been tested for sensitivity to blue light. Here we test a range of trauma associated fungal pathogens¹ and show that many, but not all, are effectively inactivated by this treatment. However, we also demonstrate that some of the antimicrobial activity previously ascribed to blue light may in fact result from secondary heating effects and thus recommend a more detailed characterization of this antimicrobial strategy prior to widespread adoption.

Results

Blue light shows antifungal activity against some, but not all, fungal species tested. For most fungal infections the initiating inocula are fungal spores and disease progression depends on germination and subsequent hyphal invasion of tissue. We therefore exposed spores of six common trauma-associated fungal pathogens (*Rhizopus microsporus*, *Mucor circinelloides*, *Scedosporium apiospermum*, *Scedosporium prolificans*,

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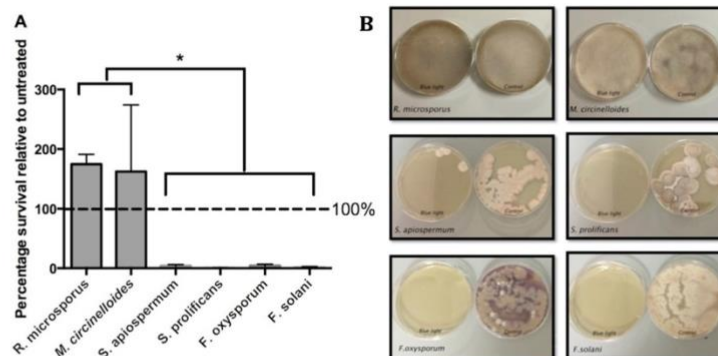


Figure 1. 60 min (216J/cm²) blue light treatment of fungal spores. Spores were inoculated in PBS treated with blue light and then plated onto appropriate agar plates for enumeration (A). Alternatively, 1000 spores were plated onto agar and then exposed to blue light for 60 minutes before being incubated for growth (B). Error bars represent standard deviation (n = 3, with three experimental replicates). One-way ANOVA followed by Tukey's multiple comparisons test shows significant difference (p < 0.05 for all comparisons) in blue light treatment survival between *R. microsporus* and *M. circinelloides* and other species tested. (B) represent images of fungal growth on agar plates following blue light treatment.

Fusarium oxysporum, *Fusarium solani*) to blue light treatment and then transferred onto agar plates for CFU counting over the following days. Blue light was highly effective against *Scedosporium* and *Fusarium* species, but showed no inhibitory effect but rather enhanced survival relative to controls on the two species of Mucorales tested (Fig. 1A).

To test the potential for blue light to decontaminate solid surfaces, we inoculated fungal spores onto agar and then treated with blue light for 1 hour at room temperature before being allowed to grow for 10 days to determine fungal survival and colony morphology. As with treatment in liquid, blue light exposure on solid (agar) media was highly effective against *Scedosporium* and *Fusarium* species, but showed no inhibitory effect on the two species of Mucorales tested (Fig. 1B). To ensure that this effect was not related to blue-light induced alteration of the agar surface structure, we performed an additional control by treating agar plates with blue light for one hour and then inoculating with fungal spores, which resulted in normal growth of all fungi tested (Supplementary Figure 2).

Germination is permanently blocked in most fungal species, but only delayed in Mucorales and *Candida*. To visualize blue light effects on fungal pathogens we performed time-lapse imaging on treated fungal spores and additionally included *Candida albicans*, which has previously been shown to be sensitive to blue light killing⁶ (Fig. 2). Time lapse imaging demonstrated that blue light treatment permanently inhibited germination of *Scedosporium* and *Fusarium* species, but that *Rhizopus microsporus*, *Mucor circinelloides* and *Candida albicans* eventually recovered full growth capability. Thus blue light induces a germination/growth arrest that appears permanent in most fungi, but only transient in the Mucorales and *Candida* species tested here. It will be of interest in the future to establish whether this pattern is conserved across the diversity of fungal species within these two groups.

Interestingly, during these studies on *Rhizopus microsporus* we also made the chance observation of a morphological change during germination into yeast-like budding (Fig. 2). Further analysis demonstrated that this budding is suppressed by exposure to light (Supplementary Figure 1). Budding of this sort has been previously reported for *M. circinelloides*⁸ but never previously observed in *Rhizopus* species, so this observation raises the possibility that budding in the absence of light stimuli may be widespread within the Mucorales.

Blue light exposure leads to secondary heating, but this is not a major contributor to the growth inhibition effect. During our investigations we noted that treated samples were significantly warmer than untreated controls. We therefore measured temperature within the medium for samples within the blue light instrument that were either exposed to blue light or wrapped within foil. In both cases, we noted a very rapid increase in temperature during instrument operation (Fig. 3). Such temperatures are likely to be deleterious to fungal spore survival and we therefore repeated our blue light treatment experiments by housing the instrument within a cold room, which limited the maximum temperature experienced to 37 °C (Fig. 4), a temperature that is fully permissive for growth of these pathogens. When we repeated this assay under these conditions, blue light retained its potent inhibitory effect on the *Fusarium* and *Scedosporium* species and, as before, showed no inhibition of Mucormycete survival (Fig. 4).

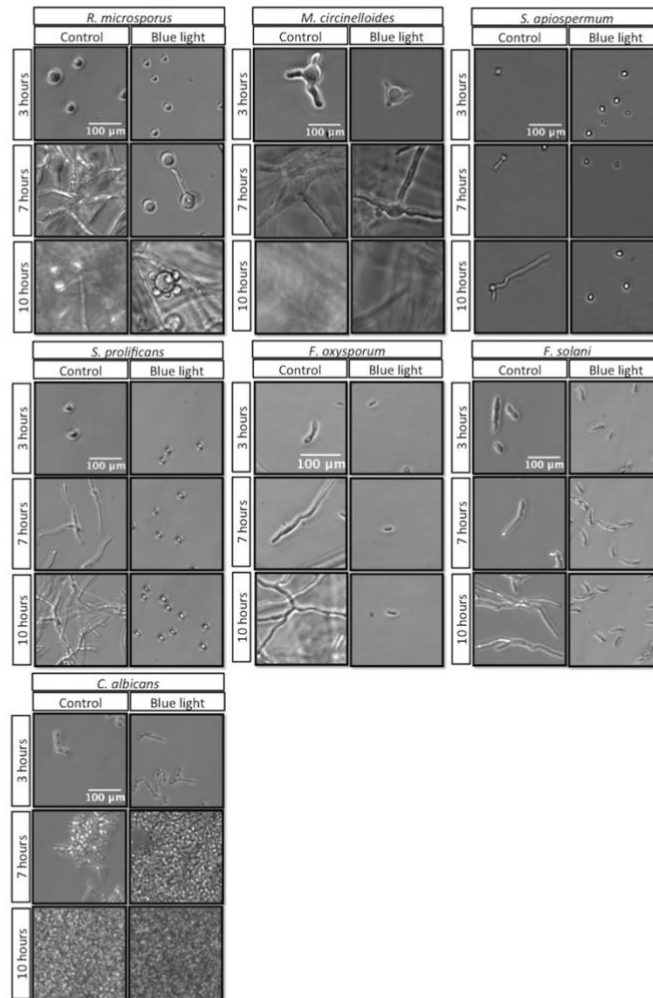


Figure 2. Visualization of blue light effect on fungal spores and cells. Time-lapse imaging shows that blue light 60 min treatment ($216/\text{cm}^2$) under controlled temperature conditions is effective against *S. prolificans* and *F. solani* species but not *C. albicans* or Mucorales.

Blue light is highly effective against pre-germinated spores, but also leads to significant cytotoxicity towards mammalian cells. In clinical settings, fungal spores might have germinated (or, in the case of *Candida albicans*, switched from yeast to form hyphae in response to environmental cues such as the presence of serum, low oxygen or high pH) and begun filamentous growth before treatment can be applied. Therefore we investigated the effect of blue light treatment on pre-germinated spores and hyphae. Spores were first germinated for 3–5 hours, and then exposed to 60 minutes of blue light (in cold room conditions to limit temperature exposure to below 37°C) followed by time-lapse microscopy (Fig. 5). In all cases, blue light treatment effectively stopped further growth of germ tubes, including in Mucormycete species, and *Candida* hyphae, two

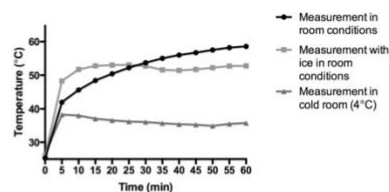


Figure 3. Temperature rises rapidly inside the blue light instrument during operation. Incubating samples on ice within the instrument is insufficient to reduce this effect, but housing the instrument within a cold room (at 4°C) during treatment maintains sample temperatures at 37°C.

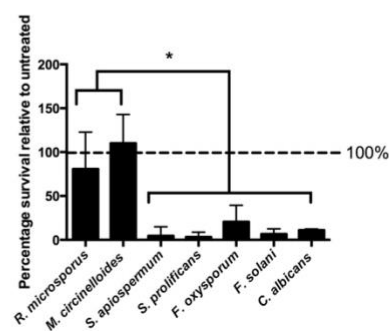


Figure 4. 60 min (216/cm²) blue light treatment of fungal spores in cold room conditions shows similar growth inhibition effects. Spores were inoculated in PBS and treated with blue light before plating for growth and CFU enumeration. Error bars represent standard deviation (n = 3, with three experimental replicates). One-way ANOVA followed by Tukey's multiple comparisons test shows significant difference (p < 0.05 for all comparisons) in blue light treatment survival between *R. microsporus* and *M. circinelloides* and other species tested.

fungal groups that show resistance to blue light as spores or yeast, respectively (Fig. 1). Thus, blue light is an effective inhibitory treatment for fungal spores that have already germinated, including for species that are resistant to such treatment as spores.

Previous work has demonstrated that blue light can also induce high levels of cell death in mammalian cells⁵. To test whether this was also the case in our system, we J774 murine cells to 15, 30, 45 and 60 min of blue light, under conditions where the temperature was controlled to 37°C. As previously reported by others for other cell types, blue light exposure led to a rapid, dose-dependent cell death in this cell line whilst J774 cells not exposed to blue light showed undetectable levels of death over the same time period (Supplementary Figure 3).

Discussion

Fungal infections are a common complication of traumatic injuries sustained in both military and civilian environments like agricultural, motor vehicle, and natural disasters or blunt crush injuries¹.

Here we have tested the most significant trauma-associated fungi, including Mucorales, *Scedosporium*, *Fusarium* and *Candida* spp. against blue light therapy. Fungal tissue infections are very difficult to treat as many of these species show intrinsic resistance to antifungals and drug accessibility to wounded tissue is poor. Thus blue light may represent a novel approach for dealing with such infections.

We have demonstrated that 60 minutes of blue light treatment, providing an equivalent total dosage of 216/cm², shows potent inhibition of fungal growth for spores that have already germinated and produced hyphae or germ tubes in all species tested. In addition, such an approach can also be an effective decontaminant of ungerminated spores for most, but not all, pathogenic species.

However, we also note some important caveats to this approach. Firstly, for resistant Mucormycete species, blue light treatment appears to counteract the effect of high-temperature and enhance subsequent germination (Fig. 1) and morphological switching (Fig. 2), the immunological consequences of which remain unknown. This likely reflects the previously characterized role of blue wavelengths as a regulator of fungal growth⁹. In an analogous context, we note that others have previously demonstrated the ability of blue light to enhance virulence

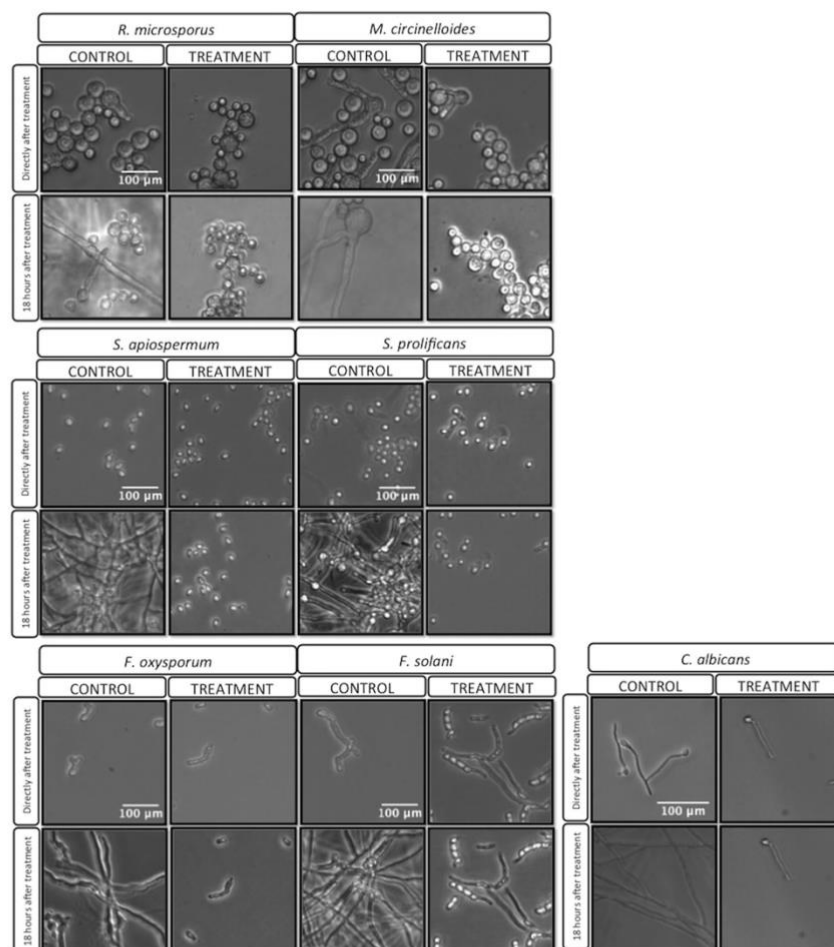


Figure 5. 60 min (216 J/cm²) blue light treatment show antifungal activity on pregerminated spores. Time-lapse imaging shows inhibition of further fungal growth after blue light treatment.

in selected bacterial pathogens, such as *Brucella* species¹⁰. Thus blue light may inadvertently work against other forms of fungal decontamination (such as heat treatment) in resistant species. Secondly, we note that the high-intensity blue light system used here generates considerable heat - a factor that is important to control for when assessing its efficacy against pathogenic microbes. The extent to which secondary heating occurs with other blue light instruments is unknown, but should be borne in mind as a potential confounding factor in other studies. Lastly, although (unlike ultraviolet) blue light is not mutagenic¹¹, both our own studies (Supplementary Figure 3) and previous work⁵ demonstrate relatively high levels of toxicity for blue light against some mammalian cells - an observation that may limit the application of this approach directly to patients. Thus blue light offers a potentially useful antimicrobial approach, but its toxicity towards mammalian cells and its limited efficacy against some fungal spores may suggest it is more appropriately used as a selective surface decontaminant than an *in vivo* antifungal.

Materials and Methods

Fungal strains. Eight different strains were tested: *Rhizopus microsporus* 12.6652333 and *Mucor circinelloides* NRRL3631¹², *Scedosporium apiospermum* IHEM 14462, *Scedosporium prolificans* IHEM 5608, *Fusarium oxysporum* IHEM 25499, *Fusarium solani* IHEM 6092, all from the Belgian Coordinated Collections of Microorganisms, as well as *Candida albicans* SN152¹³. Mucorale strains were grown on Sabouraud agar plates, *Scedosporium* and *Fusarium* spp. were grown on Potato Glucose agar plates for at least 10 days before use at room temperature, and *Candida* was grown on Yeast Extract Peptone agar plates for 1 day at 37° C.

Media. Spores were washed off agar plates with phosphate-buffered saline (PBS) (Fisher Scientific), then spun down and resuspended in PBS for cell counting in a haemocytometer. For *Candida* species, a single yeast colony was picked from YPD agar using an inoculation loop and inoculated in YPD or RPMI broth (depending on the experiment) for counting in a haemocytometer. For blue light testing, fungal spores and cells were inoculated into PBS and exposed to blue light, then transferred to appropriate agar plates after treatment for subsequent colony counting. For microscopy experiments, fungal spores and cells were inoculated in Sabouraud broth (Sigma Aldrich).

Temperature measurement. The temperature of treated cell cultures was measured within 24 well plates using a submersible aquarium thermometer (ETI, UK). The temperature was recorded constantly throughout the treatment and plotted at five minutes intervals. This temperature measurement was repeated on three separate occasions over a period of three months, with extremely consistent data on each occasion.

Treatment with blue light. A LED flood array¹⁴, composed of 144 LEDs (Henkel-Locite, Hemel Hempstead, UK), was used to treat a 10 × 10 cm area using high-intensity blue light (405 nm). All experiments on fungal cells were performed by placing samples within the treatment area for 1 hour, providing an equivalent total dosage of 216J/cm². To control for temperature effects, experiments were either performed in a cold room (4°C) or at room temperature, as described. Fungal cells were inoculated into PBS and then placed under the treatment area for 60 minutes (216J/cm²). Two control conditions were used: one plate covered with aluminum foil inside the blue light machine (exposed to temperature, but not blue light, effects) and one kept outside the instrument during operation (not exposed to either raised temperatures or blue light). Following treatment, each fungal suspension was transferred onto appropriate agar plates and CFU numbers were counted following growth (typically a few days later). Each experiment was performed in technical triplicate and repeated on at least three occasions. For determining an effect of blue light on fungal morphology assay, fungal spores were inoculated onto agar and treated with blue light for 1 hour in the room temperature then left to grow for a few days, or agar plates were treated with blue light for 1 hour, then fungal spores/cells were inoculated on it for growth. For determining blue light cytotoxicity, the murine cell line J774 was treated with blue light for 15, 30, 45 and 60 min in cold room to control for excessive heating. Following treatment cells were stained with Trypan blue for viability and counted using a haemocytometer to determine percentage survival (Supplementary Figure 3). J774 cell line was incubated in complete DMEM (Sigma-Aldrich), phenol free, plus 100U/ml of penicillin and 100 U/ml of streptomycin (Sigma), 2 mM l- glutamine (Sigma), and 10% fetal bovine serum (FBS) (Sigma) in 37°C and CO₂ condition.

Time-lapse imaging. Time-lapse imaging was performed on fungal spores immediately after treatment with blue light, using a Nikon Eclipse Ti microscope with a long working distance (LWD) 0.53 20 × objective for 18 h with 5 min intervals. Movies for publication were analysed and prepared in NIS Element software.

Light controls for *R. microsporus*. Light controls for *R. microsporus* and *M. circinelloides* NRRL3631 were performed on fungal spores that were washed off the agar plates, inoculated in Sabouraud broth (Sigma Aldrich) and covered with aluminum foil or exposed to light. Plates were incubated at room temperature for 10 hours and samples were taken for pictures.

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Author Contributions


W.J.T. designed and conducted experiments and wrote the paper. H.E.W. designed and conducted experiments. J.E.T. and R.C.M. contributed to the design and conduct of the project and writing of the paper.

Additional Information

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